Molecular Analysis of Border Disease Virus

Janice Richards

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CONTENTS

Declaration

This thesis was composed solely by myself. Most of the work presented herein was also performed by myself, where others were involved this has been clearly indicated and acknowledged, though I played a full role throughout in the design and interpretation of experiments.

JANICE RICHARDS
March 1992

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Abstract

Studies were carried out at the molecular level on Bovine Viral Diarrhoea Virus (BVDV) and Border Disease Virus (BDV). The primary objective of this work was to produce a cDNA library containing BDV sequences. BDV growth was studied and the possibility of growth enhancement using Actinomycin D was investigated, with a view to maximising virus production for a cloning experiment. BDV was grown on a large scale and 25 litres of virus in tissue culture fluid were produced. RNA was purified from this virus and used as a template in a cDNA synthesis experiment. Cloning of cDNA from the RNA was not successful, due to the lack of sufficient quantities of RNA as starting material. This stemmed from the extreme difficulty in producing enough virus, as BDV grows poorly and is difficult to purify.

Although this primary objective was not achieved, other approaches were successful. The replication of BDV was studied *in vivo* by incorporation of ^{3}H-uridine into infected cells. A time course of replication was produced.

Genomic RNA in infected cells was detected by ethidium bromide staining in agarose gels, and *in vivo* labelling of virus RNA. The RNA was shown by gel electrophoresis and rate zonal sedimentation to be of the order of 12kb in size.

Seven DNA clones produced from BVDV-NADL were used to assess homology between various pestivirus strains. One clone tested hybridised to BDV RNA in a dot-blot experiment. Northern blots using this probe confirmed the BVDV RNA to be 12kb in size in accordance with published data.

The polymerase chain reaction was used to amplify sequences from BVDV and BDV. Five different pairs of oligonucletides were assessed for their ability to prime amplification of BVDV and BDV sequences, four pairs successfully amplifed BVDV-NADL sequences and one pair amplified BDV sequences.

Finally, computer assisted sequence comparison programmes were used to analyse published sequences of BVDV and Hog cholera virus (HCV).

CHAPTER 1
REVIEW OF LITERATURE

1.1 HISTORY AND CLINICAL DESCRIPTION OF PESTIVIRUS INFECTIONS

Border Disease (BD) is a viral infection of sheep causing congenital disease and persistent infection. First described by Hughes *et al* (1959), evidence for a viral aetiology was established by in vivo transmission experiments (Gardiner and Barlow, 1972). All aspects of the disease have been discussed in a comprehensive review by Barlow and Patterson (1982). The causative virus has been classified, on the basis of physical and serological properties, in the genus Pestivirus of the Flaviviridae. The other two members of this genus are Bovine Viral Diarrhoea Virus (BVDV) which naturally infects cattle, and Hog Cholera Virus (HCV) whose natural host is the pig (Westaway *et al*, 1985). A serologically unrelated virus, Rubella virus of man, (RUV) has been grouped with the Pestiviruses under the collective term non-arthropod borne (non-arbo) togaviruses, to distinguish them from the main genus of togaviruses, the alpha viruses, which rely on an arthropod vector. Non-arbo togaviruses share an ability to cross the placenta of an infected animal and infect the foetus. The consequences of such congenital infections have been comprehensively reviewed by Van Oirschott (1983) and an exhaustive comparison of all aspects of these viruses has been published by Horzinek (1981).

The pestiviruses themselves have been the subject of several recent reviews (Littlejohns, 1989; Moennig, 1990; Collett *et al*, 1989), and their original classification as Togaviruses is now under debate (Moorman *et al*, 1990).

The clinical manifestations of pestivirus infections vary enormously in type and severity (a fact which probably reflects their complex pathogenesis). This section deals with the main clinical aspects of infection by BDV, BVDV and HCV.

1.1.1 Border disease

Since the initial description by Hughes *et al* (1959) there have been many reports both from the UK and abroad of outbreaks of this disease (Dickinson and Barlow, 1967, Osburn *et al* 1972,

Acland *et al* 1972). Outbreaks are most clinically evident at lambing time, typified by abortions, stillbirths and the birth of affected lambs. Characteristic symptoms are: generalised or localised growth retardation giving a stunted appearance, an excessively hairy birthcoat, sometimes abnormally pigmented, and varying degrees of neurological defects due to hypomyelinogenesis of the central nervous system (CNS), which can produce body tremors in the newborn lamb. Recent studies suggest that this hypomyelination could be due to depressed levels of thyroid hormones in the foetus, as a result of persistent, non-destructive infection of the thyroid gland by BDV (Anderson *et al*, 1987, 1988). These symptoms led to an alternative name for BD affected lambs: "hairy shakers". These lambs show a general failure to thrive and many will die after weaning. Those that do survive show a gradual decline in symptoms with age. These recovered animals have been termed "recovered hairy shakers" (RHS) (Barlow and Paterson, 1982). Some RHS sheep succumb to a spontaneous, acute and usually fatal disease which can develop at any time. These RHS sheep are viraemic for life. Some may succumb to a spontaneous acute and fatal disease. This disease has been termed "mucosal disease of sheep" because of its similarity to the same disease in cattle (discussed later). The main symptoms are diarrhoea and nasal and respiratory discharge (Barlow et al 1983). The onset of this disease is sudden, its course rapid (about 10 days) and the outcome is almost always death.

1.1.2 Bovine Virus Diarrhoea Virus (BVDV)

Two clinically distinct diseases of cattle, bovine virus diarrhoea (Olafson *et al* 1946) and mucosal disease (Ramsey and Chivers, 1953) were shown to be caused by serologically related viruses (Gillespie *et al*, 1961). BVD is characterised by outbreaks of diarrhoea, but has a low mortality rate. Many infections are mild, if not subclinical. Associated with such outbreaks is a rise in the incidence of abortions and stillbirths, as with Border disease. Unlike BDV infections, calves infected in utero with BVDV and surviving to term do not have characteristic clinical symptoms (Nettleton, 1985).

Mucosal disease usually occurs in young cattle and is invariably fatal. Diarrhoea, nasal discharge, depression and erosions of the GI tract are features of this disease. As with the condition in sheep, the onset is extremely sudden and the course rapid. The pathogenesis of BVDV infections has been reviewed by Brownlie *et al* (1987a).

1.1.3 Hog Cholera Virus

This virus causes a very serious and contagious disease of pigs with a high mortality rate. Also known as 'swine fever', the disease shows many features of BVDV and BDV infections, such as the ability to produce acute, chronic or inapparent infections, congenital transmission and teratogenesis resulting in stillborn offspring. HCV infections have been reviewed by Liess (1987).

1.2 PATHOGENESIS OF BDV AND BVDV INFECTIONS

1.2.1 General

Infection of adult sheep with BDV is virtually always subclinical. Following exposure to the virus, a transient viraemia occurs inducing high and persisting titres of neutralising antibodies, which cross-react with other pestivirus strains (Vantsis *et al* 1976).

The most serious and far-reaching consequences occur when BDV infects susceptible pregnant ewes. Exposure of pregnant ewes results in the same immune response as in non-pregnant animals. Nevertheless, before the virus can be eliminated by the ewe it penetrates the placenta and infects the foetus. Safe from the maternal antibodies (to which the placenta is a barrier) the virus finds a favourable environment for replication in the actively dividing foetal cells, and is free to grow without restraint.

The exact nature of transplacental transmission is not clear. The factors involved in establishment of an intrauterine infection are many and varied, and have been discussed at length

by Osburn (1988) and Pohlenz *et al* (1988). Both BDV and BVDV replicate well in placental tissue (French and Snowdon, 1974, Snowdon *et al*, 1975) and the current view is that the virus grows through the placenta. This produces lesions which, depending on their severity, may contribute to early foetal death. Although only non-cytopathic BVDV strains produce persistent infections after transplacental transmission, cytopathic strains of virus are able to cross the placental barrier (McClurkin *et al*, 1984; Brownlie *et al*, 1989). The reasons for the difference in pathogenesis of the two biotypes once inside the uterus are unclear.

The eventual outcome of a foetal infection depends on an interplay between several factors. The specific properties of the infecting virus strain, for example, virulence and cytopathogenicity, can influence the course of the infection. Likewise, the genotype of the host may modify the outcome, perhaps by its effect on the immune system (Barlow *et al*, 1980). The major determining factor though, is the age at which foetal exposure occurs, because this governs how, if at all, the foetus will respond to the virus. Foetal response in turn influences the distribution and persistence of virus, which will determine the type and extent of foetal damage.

The ovine gestation period is approximately 150 days and the foetus can first mount an immune response between 64 and 82 days (Fahey and Morris, 1978). For the sake of clarity the possible outcomes of foetal infection with BDV will be discussed in isolation and with reference to the immune-competence of the foetal host at the time of infection.

1.2.2 Infection of the immune-competent foetus

Exposure of a foetus in the last trimester of pregnancy follows much the same course as in an adult sheep. The virus is eliminated by the foetal immune response and normal virus negative lambs are born. These lambs generally have high titres of neutralising antibodies to their infecting agent, and cell

mediated reactions to the virus have also been suggested (Gardiner *et al*, 1983).

1.2.3 Infection of the immune-incompetent foetus

Exposure of a foetus to BDV before it can mount an immune response has a very much less predictable outcome.

Abortion and stillbirth can occur following early infection. If the foetus survives it may be born with classical BD symptoms or be clinically normal. There is evidence in cattle that such clinically normal animals harbour BVDV within specific cell types of the brain (Trantwein *et al*, 1987; Pohlenz *et al*, 1988). Most crucially, lambs born after exposure to BDV during this period will be persistently infected with the virus.

The birth of clinically affected "hairy-shaker" lambs has been most consistently observed following infections in the 50-63 day period. The lack of foetal immune response means the virus spreads among the tissues, and accordingly, virus is readily isolated from tissues of BD lambs (Terpstra, 1981). Persistently affected lambs have no neutralising antibodies to the virus and appear to be immunotolerant to it. This will be discussed in more detail later.

Occasionally, following infection at 50-55 days gestation, lambs have been born with severe brain malformation. Sacrificed lambs from the same experiment were shown to have severe lesions and inflammatory reactions in the CNS. The lambs which survived to term and had such malformations did not, however, display any of the classical signs of BD. Despite being exposed during a period when they were supposedly immuno-incompetent, these lambs had neutralising antibodies, and, presumably, no virus. The factors involved in producing this "alternative pathology" (AP) are not entirely clear - but virus strain characteristics such as virulence and antigenicity appear to be important. It has been suggested that a precocious immune response by the foetus may in part contribute to the lesions and inflammatory

reactions characteristic of this disease (Barlow and Patterson, 1982).

1.2.4 The persistently infected animal

As discussed earlier, persistently infected animals result from exposure of an immuno-incompetent foetus to BDV. These lambs have a persistent viraemia and no neutralising antibodies to the virus. Clinical signs may or may not be present and certainly cannot be relied upon to detect viraemic animals. Most importantly, these animals continuously shed infectious virus into the environment thus remaining a constant source of virus. They may also transmit virus vertically and produce viraemic offspring. Consequently they are of immense importance in the epidemiology of the disease. The elucidation of the mechanism(s) of viral persistence is therefore of great practical significance in this particular case, as well as representing a fascinating challenge more generally.

To establish a persistent foetal infection the virus responsible should be of low virulence so as not to kill the foetus, and low cytopathogenicity so as to minimise foetal damage caused by destruction of cells. An ability to impair or evade immune function and/or replicate in cells of the immune system are also qualities proposed by Mimms (1982). Persistence of RNA viruses in the CNS has been discussed by Kristensson and Norrby (1986).

In fact, virus recovered from persistently infected animals is invariably non-cytopathic, and only non-cytopathic strains produce persistently infected lambs after crossing the placenta.

Exposure to an antigen during foetal life is a way of producing immunological tolerance to that antigen. The unlimited replication of the virus before the foetus can mount a response would result in high levels of viral antigens being displayed to the developing immune system, with a consequent tolerance to these antigens. Persistently infected sheep

immunotolerant to BDV have a normal unimpaired immune response to other antigenic stimuli. Thus, the tolerance of BDV is not likely to be due to a generalised impairment of immune function, but rather to an inability of the host immune system to 'see' BDV antigens. This is probably due to a combination of evasion of host response by the virus, and clonal destruction of BDV specific lymphocytes during development.

The immune tolerance to BDV is specific but neither permanent or absolute (Terpstra 1981), as persistently infected animals may develop neutralising antibodies to BDV at some stage. Viraemia, however, remains present.

In experiments designed to test the specificity of this immunotolerance, in RHS sheep, Barlow *et al* (1983) showed a startling and unexpected result. RHS sheep experimentally superinfected with a cytopathic heterologous virus (BVDV) remained healthy and produced neutralising antibodies to the superinfecting virus, which was largely eliminated. RHS sheep superinfected with a homologous cytopathic virus (BDV) became severely ill with a disease clinically and pathologically similar to mucosal disease (MD) of cattle. The superinfecting cytopathic virus was readily isolated from the tissues of these animals and no neutralising antibody was detectable.

The conclusions drawn from these and subsequent experiments were firstly, that a persistent infection with BDV is a prerequisite for development of the MD-like syndrome, and secondly, that close antigenic similarity between the persistent and superinfecting viruses is necessary. It is unknown if the MD-like syndrome occurs naturally under field conditions.

Mucosal disease has been studied far more extensively in cattle than in sheep, and experimental superinfection of cattle with biologically-cloned isolates of BVDV, as well as naturally occurring outbreaks, has produced results which may shed some light on the event(s) precipitating this disease. Brownlie *et al*

(1984, 1987a, 1987b) have discussed this at length and a summary of current ideas follows.

1.2.5 Pathogenesis of mucosal disease (MD)

It is established that MD can occur only in persistently infected cattle (Roeder and Drew, 1984). A crucial question then, is what precipitates the disease. A hypothesis has been proposed by Brownlie *et al* (1984) as follows: Initial infection of a pregnant cow occurs (before her foetus is immune-competent) with a NCP form of BVDV. The calf subsequently born is immunotolerant to this BVDV strain.

At some stage, the calf encounters a cytopathic form of the same virus strain and becomes superinfected. Because the calf is specifically immunotolerant to this strain the CP virus is free to replicate without restriction in the host, and on doing so produces MD. Although this situation may occur, recent results suggest that MD may also arise despite a neutralising antibody response to the superinfecting CP virus, although the comparative efficacy of neutralising in vivo and in vitro is not known (Westenbrink *et al*, 1989).

To test this hypothesis, Nettleton (1987a) investigated the cytopathogenicity of BVDV isolates from natural outbreaks. CP virus was consistently associated with MD and with no other disease syndrome. Furthermore, superinfection experiments on persistently infected cattle showed that CP BVDV was capable of provoking MD, whereas NCP virus was not. Bolin *et al* (1985) reached similar conclusions.

There is thus substantial evidence that superinfection by CP pestiviruses is a trigger for production of MD in both sheep and cattle. A further refinement of the hypothesis refutes a criticism made against it, which was based on the fact that some workers failed to produce MD by superinfecting with a CP virus. Superinfection experiments using antigenetically different CP viruses showed that the degree of relatedness

between the superinfecting CP virus and the original NCP persisting virus is important in determining whether MD is produced (Brownlie *et al*, 1987b). Thus there must be a close homology between CP and NCP strains for the disease to occur. These authors further suggested a time dependent probability of the disease developing, depending on the degree of homology between the persistent and superinfecting viruses. Corapi *et al* (1988) substantiated this model by demonstrating that antigenic similarity between the persistently infecting NCP virus and the CP virus isolated from fatal MD cases was a consistent finding.

Having defined this working hypothesis, some questions remain unanswered. A CP virus is associated with specific molecular properties.

How do those properties relate to the diseases associated with each type? Are the two types interconvertible? Where, in fact, do CP viruses arise from? Westenbrink *et al* (1989) found that the CP virus re-isolated from experimentally induced cases of MD, was different antigenically from the original strain of CP virus used to superinfect the animal. They suggested this may be due to a mutation of the superinfecting strain, mutation of the NCP persistently infecting strain to CP, or a result of a genetic recombination between the RNAs of the persistently and superinfecting virus strains. Evidence for RNA recombination is now well established (King *et al*, 1982) and a copy choice model for producing rearrangements in the pestivirus genome has been proposed (Meyers et al 1991).

Two possibilities exist for the origin of CP viruses precipitating MD. They can be introduced from outside a herd or arise from within. Brownlie *et al* (1987b) have discounted the former idea on the grounds that the chance of introducing a virus of suitable antigenetic similarity was very slight. Also, some MD outbreaks occur in closed herds. They thus propose that CP virus arises from a NCP virus, presumably by mutation, and spreads throughout the herd, which is immunotolerant to it. No direct evidence for mutation of this sort exists. However, the

same observations which led to the hypothesis of antigenically similar superinfecting strains, can also be invoked in support of the mutation theory.

The very fact that only antigenically similar pairs of viruses are isolated from cases of MD suggests that one may have arisen from the other. Pairs of CP and NCP viruses from cases of MD have been compared at the protein level (Pocock *et al*, 1987) and found to be very similar.

The first part of this introduction has dealt with both clinical discription and pathogenesis of BVD and Border Disease. The latter will review the properties of the viruses causing these diseases.

1.3 PROPERTIES OF THE VIRUSES

While the clinical and pathological aspects of pestivirus infections are reasonably well represented in the literature, there is a dearth of information on the actual properties of the viruses. Most of the information which is available relates to Bovine Viral Diarrhoea Virus (BVDV), and, to a lesser extent, HCV. The few reports concerning border disease virus (BDV) are limited to morphological and serological findings. At the present time, there is apparently only one report in the literature dealing with the molecular organisation of BDV (Moerlooze *et al*, 1990).

1.3.1 Classification

The family of togaviruses was established to accommodate small enveloped viruses with icosahedral symmetry and a positive sense RNA genome. Initially it consisted of two well-defined groups; the alphaviruses and flaviviruses, both of which require an anthropod vector. Horzinek (1981) initially coined the term pestiviruses as a generic name for HCV and BVDV, which, though sharing the structural properties of togaviruses, were antigenically unrelated to the existing genera.

Horzinek (1981) defined the togaviruses as follows: "Spherical 40-70nm in diameter and consisting of an isometric probably icosahedral, nucleocapsid, tightly surrounded by a lipoprotein envelope. The viral membrane contains host cell lipid and one to three virus specified polypeptides, one or more of which is glycosylated. The nucleocapsid is constructed from one non-glycosylated polypeptide containing a single colinear molecule of single-stranded RNA (molecular weight about 4×10^6 daltons) which is infectious when extracted and assayed under appropriate conditions".

Recently, the validity of such a structural classification has been questioned (Horzinek, 1987). Flaviviruses have recently been elevated to family status as disparity between their replication strategy and that of the alphaviruses has emerged. Molecular organisation of the genome and replication strategies are now considered more relevant for taxonomy than physical structure. Molecular information on the pestiviruses has revealed that they are more similar to flaviviruses than to alphaviruses and there is currently debate over their taxonomic status (Collett *et al*, 1989).

1.3.2 Morphology and physicochemical properties

A wide size range has been reported for BVDV ranging from 30 to over 100nm. Ritchie and Fernelius (1969) observed 50nm diameter particles. The more recent studies have tended to be in agreement, with one exception: Chu and Zee (1984) showed particles 120 +/- 30nm in diameter in infected cells. These particles consisted of a core structure surrounded by a membrane 5-7nm thick, which possessed numerous projecting spikes 4-5nm in diameter. Contradictory reports regarding virus size have been published (Ohmann and Bloch, 1983; Gray and Nettleton, 1987). The former of these reports detailed oval and round particles 40-55nm in diameter consisting of a core structure surrounded by a smooth membrane. Gray and Nettleton reported similar sized 46nm particles with a core structure, associated with rough endoplasmic reticulum (RER). These authors also published the

first ultrastructural details of BDV, which appeared to be morphologically indistinguishable from BVDV.

In the report by Ohmann and Bloch (1983) mature viruses were never observed free in the cytoplasm, but in distinct vesicular structures. They proposed that viral structural components never exist free in the cytoplasm and that morphogenesis takes place entirely within these vesicles. An important conclusion from their study was that mature BVDV virions do not appear to leave the host cell by "budding" or by cytolysis. Instead, they appear to escape by exocytosis, the virion containing vesicles fusing with the cell membrane to release their contents. Presumably, on cell destruction late in the growth cycle the vesicles will disintegrate and release the remaining virions.

Border disease virus has been studied even less. Gardiner et al (1972) reported it to be 27nm in diameter. Later reports confirmed this and showed it was a morphologically similar particle to BVDV (Vantsis *et al* 1976; Harkness and Vantsis, 1982; Gray and Nettleton, 1987). The hydrodynamic properties of both viruses are very similar. In comparative experiments, BD-2 virus and BVDV (NADL) were centrifuged in isopycnic gradients. Both were shown to have a buoyant density of 1.108g/ml, BD-M possessed a buoyant density between 1.09 and 1.18g ml in 10-35% sucrose gradients. This value was similar to that quoted for BVDV (NADL) under comparable conditions (Stott *et al* 1974). Laude and Gelfi (1979) showed that the buoyant densities of BDV, BVDV and HCV were all identical in sucrose gradients.

BVDV has been shown to be sensitive to heat, (56^{o}C, 30 min) ether and chloroform (Gillespie *et al* 1961; Tanaka *et al* 1968). It was shown to have a single-stranded RNA genome (Hafez and Liess, 1972) which is directly infectious (Diderholm and Dinter, 1966a).

Similar sensitivities were attributed to BDV (Vantsis *et al* 1976). No report has demonstrated the infectivity of the BDV RNA.

Thus BDV and BVDV are indistinguishable on the basis of physico-chemical criteria. Along with HCV, they form a structurally homogeneous group within the Togaviridae (Laude and Gelfi, 1979).

1.3.3 Virus-specified proteins

Only limited information (Akkina 1983, 1990) is available on the protein composition of BDV, and the following discussion refers therefore to BVDV throughout.

The first report of analysis of BVDV (associated) proteins was by Pritchett and Zee (1975). These authors analysed labelled polypeptides from virus-infected cells. Four reproducibly migrating electrophoretic zones were obtained, with molecular weights of 25K, 70K, 50-59K and 93-110K. Within each zone, distinct peaks were clear, which could have been separate, closely migrating proteins, though the authors concluded that this was unlikely due to the heterogeneous migration pattern of the peaks. They proposed instead that the peaks represented differences in carbohydrate moieties of the proteins.

Mattaeheus (1979) demonstrated three major polypeptides of molecular weights 55K, 44K and 34K, the larger two being glycosylated. Coria *et al* (1983) produced results in formal agreement with Pritchett and Zee (1975). Four polypeptides were detected, of molecular weights 25K, 54K, 66K and 75K, with the 54K and 75K polypeptides being glycosylated. Akkina (1983) demonstrated five polypeptides specific for BVDV by radio-immune precipitation (RIP). These had molecular weights 115K, 80K, 54K, 45K and 35K. These results were duplicated almost exactly

by Purchio *et al* (1984a) using the same method. The former report also contains the only known reference to the molecular structure of BDV - using BVDV antiserum in RIP, two BD specific proteins of 115k and 60k were detected. Peptide mapping analysis showed the BD and BVDV 115k proteins to be similar.

The two most recent reports seem at first sight to have further complicated the issue, demonstrating eight and twelve putative viral proteins respectively (Pocock *et al* 1987; Donis and Dubovi, 1987a). However, at the lower end of the molecular weight range, they are in excellent agreement with each other.

For ease of comparison the results from the publications discussed are summarised in Table 1.1

Donis and Dubovi (1987a) used both direct analysis and RIP to detect BVDV proteins. By direct analysis they detected the twelve polypeptides detailed in Table 1.1. Their rationale was that visualisation of all BVDV proteins may be hampered by the specificity of the antiserum as encountered in RIP analysis. Unfortunately, in abandoning such specificity, there remains no means of positively identifing the visualised proteins as virus specified. Recognising this dilemma, the authors minimised host-cell protein synthesis as far as possible, by inhibitory treatments including actinomycin D and hypertonicity. Under such conditions, RIP failed to detect some polypeptide species shown by direct analysis. This may be interpreted either as successful discrimination of virus-specific proteins, or as bias against poorly immunogenic (but still virus-specific) proteins.

Table 1.1

BVD virus-specific polypeptides

Strain	NADL[a]	NADL[b] C24V	Singer[c]	NADL[d]	NADL[e]	Singer[f]	Pestivirus[g]
						165	
						135	
	93-100*			115	120	(gp118)	p125
				80	87	80	p80
	70		gp75			(gp75)	
			66		gp69	(gp65)	gp62
	50-59	gp57	pg54	gp55	gp57	gp57	gp53
		gp44		(45)	gp49	gp48	gp48
		34		(38)	37	37	
					33	32	
	25		26		gp23	(gp25)	gp25
						19	p20

*MW values x 10^3. Numbers in parentheses represent minor components.
[a]Pritchett and Zee (1975), [b]Mattaeheus (1979), [c]Coria *et al* (1983), [d]Purchio *et al* (1984a), [e]Pocock *et al* (1987), [f]Donis and Dubovi (1987a,b), [g]Collett *et al* (1988b).

Taken from V. Moennig, Vet. Micro., 23: 35-54 (1990).

An important point arising from this work is the danger in any sort of quantitation of results, even relative comparisons. The degree of labelling of a particular protein varied with the precursor used, i.e. different proteins were preferentially labelled with different precursors. Also, bias towards immunodominant epitopes by RIP may misrepresent the relative proportions of each polypeptide.

In a companion paper to the above, the same authors describe analysis of BVDV glycoproteins by one and two dimensional gel electrophoresis (Donis and Dubovi, 1987b). They detected six virus-induced glycoproteins, the most abundant of which were 48k and 56 to 58k. The 56k protein migrated heterogenously in one dimensional gels. Following digestion with Endoglycosidase F, which removes oligosaccharide, this protein migrated as a single-band of 52k, thus apparently losing 4-6k in carbohydrate side chains. This suggested differences in migration patterns are due to variations of glycosylation.

The functional significance of most of the proteins identified is unclear. However, the 56K glycoprotein (which is the only protein consistently identified by all published reports) appears to be a constituent of the viral envelope, and involved in neutralisation (Donis *et al*, 1988). The 48k glycoprotein has also been suggested as an envelope component. It is of course unlikely that all twelve proteins identified are virion structural components, even allowing for the fact that some may be glycosylation variants of others. Some may be non-structural virus-induced proteins, while others may have a precursor/product relationship. Recent studies at the molecular level have shown the likely processing pathways of pestivirus polyproteins (Collett 1992).

In summary, the results of reports to date on the structural and virus-induced polypeptides of BVDV are shown in Table 1.1. The variation in the observed molecular weights is probably due

to differences in experimental design and method combined with the use of different virus strains. In this context it is relevant to recall the results of Pocock *et al* (1987) who showed considerable variation in migration patterns in SDS PAGE of BVDV proteins from different virus strains.

There seems, from Table 1.1 to be a general consensus of the following BVDV proteins: VP1 115K, VP2 80K, VP3 55K, VP4 48K and VP5 37K. There is also evidence for a small 25K protein. NCP Border disease virus apparently has a similar 120K protein, and proteins of 130K and 80K have been demonstrated in CP BDV infected cells (Dutia *et al*, 1990).

Only by direct sequence analysis of the genome and in vitro translation experiments will all the possible protein products be unequivocally identified. This will be further discussed in considering the molecular biology of the virus.

1.3.4 Antigenic relationships between pestiviruses

The pestiviruses are serologically unrelated to any other members of the togaviridae, including RUV (Porterfield *et al*, 1978).

The serological relationship between HCV and BVDV has been studied by a variety of tests. A common soluble antigen (SA) was shown by immunoelectrophoresis (IEF) (Mattaeheus and Van Aert, 1971). This soluble antigen seems to be a non-structural viral glycoprotein which appears early in the infectious cycle, before the mature virions (Mattaeheus, 1980). Its early appearance makes it likely to be a structural precursor or a virus encoded enzyme.

Plant *et al* (1971) confirmed the relatedness of BVDV, BDV and HCV by immunodiffusion (ID). All pestiviruses cross react in immunofluorescence (IF) tests, probably due to the common SA. Neutralisation tests however are more stringent, and can distinguish BVDV and HCV, and also different strains of BVDV. Gillespie *et al* (1961) showed that different BVDV strains were antigenically close but distinguishable.

With the BD viruses, two strains, BD-M and BD-2, were shown to be more related to each other than to BVDV (NADL) and more related to NADL than to HCV (Laude and Gelfi, 1979).

Nettleton (1985) investigated the relationship between several CP BVD strains, and CP and NCP BD strains and reached the following conclusions. In neutralisation tests, the BD-M strain was distinguishable from four BVDV strains to the extent that it may be designated as a separate serotype. This confirmed and extended the findings of Vantis *et al* (1976): all four BVDV strains were very closely related, but distinguishable. IF tests failed to distinguish any BD or BVD viruses tested, thus demonstrating a common antigen detected by this test which is not involved in virus neutralisation.

Western blotting experiments detected a 77-81K protein in all BD and BVD strains examined. This was almost certainly the common SA of the pestivirus group. A smaller 35K protein was detected only in BVDV preparations with homologous antisera. This protein (VP5) has previously been suggested to be type-specific (Mattaeheus 1981) and may possess epitopes involved in virus neutralisation.

Peters *et al* (1986) used monoclonal antibodies in a preliminary characterisation of BVDV specified proteins. Several interesting results emerged from this study. Firstly, all CP and NCP BVD viruses along with HCV share a common epitope as shown by an antibody reacting with all of these. Secondly CP and NCP strains appeared to be antigenically distinct, and the epitopes concerned have been mapped to a 79K protein by western blotting. Furthermore, antibodies which failed to react with NCP BVDV (but reacted with CP) also failed to react with HCV which is NCP. No attempt was made to map the epitope concerned on HCV.

The most recent report has extended the use of monoclonal antibodies to classify BVDV strains on the basis of virus neutralisation tests (Bolin *et al* 1988). A panel of nine antibodies was used, all of which detected only the 56-58K glycoprotein. Three separate epitopes appeared to be involved in the neutralisation function. One was conserved between all strains examined and appeared to be the main one involved in neutralisation. Another was similarly broadly distributed but was only weakly involved in neutralisation. A third epitope, on this protein allowed the classification of groups of viruses. Apparently CP and NCP strains were not distinguished by their 56K glycoprotein, and therefore not by neutralisation tests. Four groups of BVDV viruses were classified in this way, the NADL strain was the only member of its group. Thus the 56K virus membrane glycoprotein appears to be the main determining factor in antigenic variation.

Bolin *et al* (1988), Bolin and Ridpath (1990) have studied the neutralising activity and protein specificity of antibodies induced by vaccination of persistently infected cows with a cytopathic (NADL) BVD virus. Antisera were collected during a period of 12 weeks following exposure of persistently infected animals to the NADL virus, and tested for their neutralising activity against various cytopathic (CP) and non-cytopathic (NCP) strains of BVDV. As expected, the resultant antisera neutralised some BVDV strains but not others, including the homologous NCP persistently-infecting strain. No subsequent clinical data were presented, but it would be interesting to know if superinfection by the non-neutralised strains was followed by clinical mucosal disease.

These authors also studied the time course of appearance of antibodies specific for each BVDV protein, by radioimmune precipitation (RIP) of lysates of cells infected with various BVDV strains. The first antibodies to be produced against those strains which were neutralised were directed against the 56k protein. Antibodies to the 115K, 80K and 47K proteins were not

detected until 7 weeks after exposure. In contrast, when control animals free of a persistently infecting virus were exposed to NADL, antibodies specific for these proteins appeared within 2 weeks. The delay in generating antibodies to these proteins in persistently infected animals is probably due to their highly conserved nature, which would mean that they are not sufficiently different from those of the persistently infecting strain to overcome the immune tolerance of the host. This immune tolerance, however, is clearly not absolute; antibodies generated following exposure to the 115K protein detected after 7 weeks will also recognise the 115K protein of the persistently infecting strain. Furthermore, at 12 weeks after exposure to NADL, antibodies to the more significant 53K protein of the persistently infecting strain were detected. These antibodies did not possess neutralising activity. Thus, antibodies raised against one strain of BVD can react with the 56K protein of a second strain, without neutralising this second strain. This implies that there are structurally conserved epitopes on the 56K protein which are of little or no importance for virus neutralisation, and/or that the neutralisation epitope(s) are altered in the second strain, allowing binding but not neutralisation.

The work of Magar *et al* (1988) further supports this idea. These authors studied the reactivity of one monoclonal antibody (raised against NADL 56K protein) in immunofluorescence (IF) and neutralisation tests against a panel of BVDV strains. They found that BVD viruses (both reference and field strains) could be classified according to their reaction with this monoclonal antibody (Mab). One group were positive in IF and neutralisation tests, the second group were positive in both tests. The most interesting group, however, comprised strains which were clearly positive in IF tests but which were not neutralised by the Mab. This suggests that for these strains, the epitope is altered, such that the Mab can still bind (as shown in IF tests) but not with sufficient avidity to effect neutralisation. A second interesting result from these experiments was that the

degree of fluorescence varied (consistently) between strains in the IF test. This was suggested to be due to different amounts of expression of the 56K protein in different strains, and not differing levels of virus replication, since polyclonal antiserum produced uniformly bright staining of all strains.

In summary, pestiviruses have substantial antigenic homology. A common soluble antigen (SA), which is probably a 70K-80K non structural protein has been detected. NCP viruses appear to lack an 80K protein or have an antigenically different form of it. A 35K protein has shown limited evidence of being type specific, but the bulk of antigenic variation appears to occur in the 56k glycosylated membrane protein.

1.4 VIRUS REPLICATION IN THE INFECTED CELL

1.4.1 Growth in tissue culture

The majority of studies on BVDV and BDV have used primary or low pass secondary cell cultures in which to propagate the viruses. A variety of cell types has been shown to be susceptible to BVDV (Horzinek, 1981). In particular, low pass secondary bovine embryonic kidney (BEK) cells have been widely used (Gillespie *et al* 1961) but bovine embryonic spleen or testis cells have been equally useful (Horzinek, 1981).

BDV has been cultivated in both ovine and bovine cells, for example bovine embryonic testis or foetal lamb kidney (FLK) cells. One strain of BDV has shown poor growth in BEK cells but was grown successfully in FLK cells (Vantsis *et al* 1976).

A major problem with the use of bovine and ovine secondary cultures is the sporadic occurrence of contamination by latent NCP pestiviruses. Foetal bovine serum used as a growth supplement has also been shown to be contaminated by pestiviruses. Various rates of contamination have been quoted, some as high as 100%. Heat inactivation can greatly reduce but not eliminate the problem (Nuttall, 1978).

The possibility of contamination by NCP pestiviruses should therefore be borne in mind when considering experiments conducted before recognition of the problem. Some workers have reported the use of pestivirus-free cell lines to grow BDV and BVDV (Laude and Gelfi, 1979, Potts et al 1982).

The growth cycles of all three pestiviruses have been studied, but to a fairly limited degree. Nuttall (1978, 1980) has reported comparative growth studies between CP and NCP strains of BVDV in calf testis cells under presumed single cycle conditions. The kinetics of replication of both strains were similar; a lag phase of 6 to 8 hours followed by an exponential phase of up to 12 hours post infection with a subsequent plateau. During the exponential phase, the titre of the intracellular virus approximately equalled that of the released virus, when the plateau was reached, extracellular virus exceeded intracellular virus by about 0.5 log for the CP and 1.0log for the NCP strain.

The overall yield per infected cell was 58 times less for the NCP strain, although the actual rate of replication was not appreciably slower. The maximum titre reached was about 10^6 pfu/ml.

This author also studied the effects of the drug actinomycin D (ACD) on the replication of BVDV, on the basis that this drug had been reported to enhance the replication of other RNA viruses (Heller, 1963). Surprisingly, CP BVDV replication was inhibited (by 30-100%), whether this inhibition was maintained throughout the growth cycle or confined to the time measured (20 hours post infection) was not investigated. The former would seem most likely on the basis of the report by Aynaud (1968) who observed a similar enhancement of HCV replication during the exponential phase in cultures treated with ACD; but the enhancement was transient, and a final reduction in virus yield was observed.

The growth curves of a CP strain of BDV have been compared to those of two CP BVDV strains in foetal lamb brain cells (Nettleton, 1985). There were no significant differences in the growth curves, which were exponential until about 20 hours post infection, followed by a plateau which showed no signs of falling off by 120 hours post infection.

Though CP strains of BVDV appear to replicate much better than the NCP ones (Nuttall, 1980) this does not apply to BDV where both strains grow at comparable rates (unpublished observations).

One problem in studying togavirus growth, which has emerged from an extensive review on the subject (Horzinek, 1981) is the fact that infection of cells in a culture may be asynchronous, with a low number of cells becoming infected initially, regardless of the input m.o.i. This has been observed for RUV (Wong *et al* 1969) and EAV (Van der Zeijst, 1975 cited from Horzinek, (1981) among others. None of the studies on BVDV growth has included infectious centre assays at the initial stage to confirm the percentage of cells infected at the outset. Nuttall (1980) showed that 100% of cells were infected by 48 hours post infection, but this would not guarantee single cycle conditions at the outset.

Thus, studies on the kinetics of pestivirus replication have perhaps been substantially compromised by a poor efficiency and asynchrony of infection.

1.4.2 Viral RNA synthesis

Of the togaviridae, replication of both alphavirus and flavivirus, now flaviviridae, (Horzinek, 1987) is well defined and a number of studies have been done on RUV (Hovi, 1972, Sedwick and Sokol, 1969, Wong *et al* 1969).

Reports on BVDV RNA synthesis in the infected cell are few. Nuttall (1978) followed the synthesis of viral RNA by

incorporation of labelled uridine. By comparing the difference in incorporation between infected and uninfected cells, an estimate of the amount of virus-induced RNA synthesis was obtained. However, this did not discriminate between authentic viral replication and host cell transcriptional responses (either positive or negative) to infection.

Viral RNA synthesis was first detectable 7 hours after infection. Having demonstrated the incorporation of label into viral RNA, this author surprisingly failed to detect such radiolabelled RNA in progeny virions. Moreover, in the latter half of the replication cycle, all the previously synthesised labelled RNA was abruptly degraded. The replication of positive stranded RNA viruses requires the synthesis of a negative template strand which is never incorporated into the virion. The author's suggestion was that all of the label had been incorporated into negative strands such as this, which were presumably degraded after serving their function.

In a comparative study (Karrar, 1983) RNA synthesis of CP and NCP isolates of BVDV was examined. Maximum levels of synthesis occurred 12-24 hours after infection, and CP viruses showed a significantly higher level of RNA synthesis than NCP strains, though the incorporation of this labelled RNA into virus was not tested.

Renard *et al* (1985) have demonstrated the incorporation of labelled uridine into a full length viral RNA by 12 hours post infection.

There are no reports of BDV RNA synthesis published at this time.

1.4.3 Cytopathogenicity

The term "cytopathic effect" (CPE) describes virus-induced cell damage, whether morphological or biochemical. However, the mechanism by which a virus causes such effects still remains

obscure. CPE is typified by cytoplasmic vacuolation, pyknotic nuclei, cell rounding and detachment, and eventual cell death, though the type and severity of CPE depend on virus strain and cell type. BVDV and BDV strains have been classified according to their ability to produce these effects, and the distinct roles in pathogenesis assigned to CP and NCP strains imply that this division is a pertinent one.

There is evidence, however, that cytopathogenicity or its lack may not always be an intrinsic property of a virus. The CPE produced by some strains of BVDV have been shown to be modified or eliminated depending on the cell system used (Fernelius and Lambert, 1969; Johnson and Rosenbusch, 1990). Similarly, certain BDV strains were CP for FLM cells but NCP for calf testis cells, (Laude and Gelfi, 1979) and the failure to develop CPE was not due to a failure to replicate the virus. Thus permissiveness for viral replication does not invariably coincide with susceptibility to CPE.

Despite the above variations, for most strains cytopathogenicity or its lack appears to be a stable phenotypic trait, the basis of which has not yet become clear. Few comparative studies on the virology of CP and NCP strains have been published, the majority of those that have deal with the clinical and pathological effects of each type. The main differences observed between CP and NCP strains will now be outlined.

CP strains consistently show higher rates of replication than NCP strains. Nuttall (1980) showed a 58 fold increase in the yield of infectious virus per cell for CP BVDV strains. Cells infected with CP strains also showed strong fluorescence in IF tests whereas those infected with NCP strains stained poorly, also suggesting a higher concentration of viral antigens in the CP virus infected cells (Nuttall, 1978). A similar observation was made by Mahnel and Van Moreau (1984) who found strong fluorescent antibody staining of CP BVDV virus infected cells

but not with NCP virus infected cells. These authors also showed a distinct and characteristic growth cycle for each strain, the CP strain producing high amounts of virus in a short time and the NCP virus slowly rising over a longer time to reach a similar final titre. Karrar (1983) has demonstrated higher levels of virus-induced RNA synthesis in cells infected with CP BVDV than with NCP virus.

A second difference in tissue culture is the ability to induce interferon synthesis. CP strains will induce interferon but NCP strains apparently lack this capacity, as judged by their inability to suppress heterologous superinfection (Diderholm and Dinter, 1966b).

However, NCP BVDV can interfere with the replication of CP strains in tissue culture (Gillespie *et al* 1961). Such interference has not yet been conclusively demonstrated in vivo.

The fluorescence studies already discussed imply that NCP strains of BVDV have a much lower rate of synthesis of viral antigens, and it has been shown that buffy coat cells from a persistently infected animal do not display viral antigens on their surface, despite replicating the virus (Nuttall, 1978). In vivo, such a mechanism may contribute to evasion of the host immune response.

There have been few structural comparisons between the CP and NCP biotypes of BVDV. Pocock *et al* (1987) demonstrated the first biochemical difference between CP and NCP biotypes. All NCP types examined were characterised by the absence of an 87K protein which was always present in CP BVDV infected cells. This protein appears to be a derivative of a 120K protein and the authors suggest that NCP strains may have a defect in the processing of the 120k precursor, though a corresponding increase in the amount of this precursor in NCP virus infected cells was not shown. The absence of an 80K protein in NCP virus infected cells has since been confirmed by several reports

(Peters *et al* 1986; Donis and Dubovi, 1987a; Magar *et al* 1988) Donis and Dubovi (1987b) also confirmed the relatedness of the 118K and 80K polypeptides, and demonstrated that the former was far more abundant in NCP virus infected cells than in those infected with CP virus.

Previously it has been suggested that a mutation causes conversion of an NCP strain to a CP strain (Howard et al 1987). The processing of the 118K polypeptide by CP strains is not due to an extra proteolytic activity absent in NCP strains (Donis and Dubovi, 1987b). These authors therefore suggest that the difference in processing is due to the presence of a protease sensitive site in the 118K protein of CP strains, allowing degradation to the 80K protein by cellular proteases.

Monoclonal antibodies can define structurally CP and NCP strains of BVDV (Peters *et al* 1986). This epitope has been mapped by western blotting to a 79K protein which is probably the same protein shown to be lacking in NCP strains by Pocock *et al* (1987) and Donis and Dubovi (1987b).

Thus, the basis of cytopathogenicity is still largely unresolved. There seems to be a general consensus that CP BVDV strains replicate faster than NCP strains, though this is not necessarily reflected in higher virus titres from CP strains (Donis and Dubovi, 1987b). However, CP and NCP strains of BDV replicate at similar rates (unpublished observations).

It has been postulated that faster replication results in the accumulation of double-stranded replicative intermediate RNA which is toxic for host cells. This view is supported by the fact that double-stranded RNA is a potent interferon inducer. CP strains induce interferon, whereas NCP strains do not. The biochemical difference, in which NCP strains lack an 80K protein present in CP strains, is interesting; if we accept that NCP strains do have a lower replication rate, then it could be suggested that lack of the 80k protein is a rate-limiting

factor, and that this protein is therefore involved in replication of the viral RNA. Donis and Dubovi (1987b) have proposed a similar hypothesis in which the 118k protein has a negative regulatory effect on transcription or the 80k protein enhances it. It is equally possible that both mechanisms occur.

Thus, the classification of pestivirus strains as CP and NCP seems to represent a natural dichotomy within the group, which is pivotal in determining the behaviour of the virus in vivo and in vitro. The molecular basis of the distinction has been defined for some strains of BVDV (Meyers et al 1991).

1.5 MOLECULAR BIOLOGY OF PESTIVIRUSES

Until recently, there was a great lack of information regarding the molecular biology of pestiviruses, probably due to the extreme difficulties involved in obtaining the large amounts of pure virus necessary for analysis. Only BVDV, and recently HCV, have been fully characterised at the molecular level and the following discussion will be largely confined to BVDV.

1.5.1 Characterisation of the genome

The genome of BVDV was first shown to consist of RNA in 1966. This RNA was shown to be directly infectious (Diderholm and Dinter, 1966a).

Prichett *et al* (1975) published the first attempt to characterise the BVDV genomic RNA. These authors investigated whether size heterogeneity of the virus was reflected in a similar (physical) heterogeneity of the RNA. Purified virus containing labelled RNA was prepared, and the nucleic acid was analysed by isopycnic centrifugation and polyacrylamide gel electrophoresis. By both techniques one major and two minor RNA species were detected, the sedimentation coefficients in high ionic strength were 38s, 31s and 24s, corresponding to molecular weights of 3.22, 2.09 and 1.22 x 10^6kd respectively.

The two minor components were suggested to be incomplete genomes as the sum of their molecular weights approximately equalled that of the major component, although they could have been degradation products.

Felmingham and Brown (1977) showed that extracted RNA from purified virions was homogenous, banding at 40s in sucrose gradients. They also showed this RNA to be single-stranded. RNA isolated from infected cells, however, apparently revealed a 20s component in addition to the 40s one. Size analysis on polyacrylamide gels showed these RNAs to be 3.6 and 7 x 10^6kd. The larger RNA was double-stranded as judged by its resistance to RNAase, and the authors suggested it represented a replicative form of the virus.

Other authors dispensed with the analysis of purified virion RNA and instead analysed RNA synthesis in the infected cell. Purchio *et al* (1983) detected a single 8.2kb RNA in cells infected with BVDV, 24 hours after infection. The size was determined using formaldehyde agarose gels. Sedimentation properties of this infected-cell-specific RNA were analysed in formaldehyde gradients and a sedimentation coefficient of 33s was obtained.

Recently, the genomic RNA of the Osloss strain of BVDV has been cloned and sequenced (Renard *et al* 1985, 1987a,b,c). RNA from purified virions was shown to be 12.5kb in size and lacked a poly A tail. RNA from infected cells showed a similar size. No subgenomic RNA was identified.

These authors prepared a cDNA library and eventually obtained clones which were characterised by restriction mapping and cross hybridisation, and shown to span the entire BVDV genome. Sequencing of these clones provided the entire nucleotide sequence of the genome (Renard *et al* 1987a).

The genome length was 12490bp. Two large open reading frames (ORFs) were identified which were non-overlapping. The first ORF does not commence with the first AUG in the sequence. The second ORF is separated from the first and terminates before the end of the RNA leaving a 3' non-coding region. The two ORFs must code for large polyproteins which are processed to give the viral polypeptides. The predicted amino acid sequence of these polyproteins was analysed by hydrophobicity plots. The first polyprotein begins with a highly charged positive region corresponding to a protein of about 28K which is suggested to be the virus capsid protein. This is followed by two putative membrane glycoproteins of 45-50K (in their unglycosylated form). Thus the first polyprotein appears to code for the structural proteins of the virion. The second reading frame is less distinctive and was suggested to code for the non-structural proteins. It is now recognised that Osloss has only one long ORF, and not two.

Following the report of the cloning of the BVDV-Osloss sequence, a second group have reported the cloning and sequencing of the NADL strain of BVDV (Collett *et al* 1988c). This group prepared RNA from cells infected with BVD NADL and purified the viral RNA by taking advantage of its secondary structure. By using a lithium chloride precipitation technique, the viral RNA was retained in the supernatant fraction, with only some low molecular weight RNAs as contaminants.

The purified NADL RNA was shown to be 12-13kb in size as determined by its migration on methyl mercuric hydroxide gels, confirming previous reports. The RNAs of other CP and NCP strains of BVDV showed identical electrophoretic mobilities. Sequencing of the cDNA clones provided the entire nucleotide sequence of the NADL strain. This sequence contained only one long open reading frame (ORF) in contrast to the 2 ORFs reported for Osloss. This ORF consisted of 3988 codons, potentially coding for a 449K giant polyprotein. The amino acid sequence of this polyprotein produced by translation of the ORF was analysed in a companion paper to the above (Collett *et al* 1988b). Fusion

proteins from each representative area of the genome were synthesised, and antibodies to these fusion proteins were used in immunoprecipitation analyses to identify the authentic BVDV proteins. In this way 83% of the genome was assigned a coding function.

The first protein appears to be a 270 residue protein rich in proline and lysine residues, and presumed to be the virus capsid protein. The next segment codes for a 116K precursor which yields 3 glycoproteins gp53, gp48 and gp25. gp53 is the presumed membrane glycoprotein with important neutralisation sites.

The next identification region is that coding for p125 which in cytopathic strains is processed to p80 and p54. The final part of the coding sequence specifies a 133k protein which is processed to p58 and p75. p75 is the presumed viral RNA polymerase, or at least a component of it.

As discussed previously, pestiviruses appear to be more closely related to flaviviruses than to togaviruses. Like pestiviruses, flaviviruses also have a genome of positive sense containing one large ORF. Comparison of the NADL nucleotide sequence with several flaviviruses over the entire genome revealed virtually no homology. Comparison of the overall amino acid sequences of the translated ORFs also failed to reveal any extensive homology (Collett *et al* 1988a). However, comparison of the organisation and features of the protein coding regions was more rewarding. The order of gene products appears to be very similar in both viruses, ie capsid protein, glycoproteins, followed by non-structural proteins ending with the viral RNA polymerase.

The sequences of two HCV strains have also recently been reported (Moorman *et al*, 1990; Meyers *et al*, 1989) and the homology to the BVDV strains is quite high, around 85% at the amino acid level.

1.5.2 Expression of the Osloss genome

The 8.2kb RNA described by Purchio *et al* (1984b) has been translated in vitro using a reticulocyte lysate system. On denaturing the RNA, a large spectrum of polypeptides was translated ranging from 50K to 150K. None of these co-electrophoresed with authentic viral proteins. Isolating polysomes from BVDV infected cells however gave translation products of 115K and 80K which did co-migrate with viral proteins.

Renard *et al* (1987b) have expressed virtually the entire coding sequence of this virus as B-Galactosidase fusion proteins in *E. coli*. The immunoreactivity of only one of these fusion proteins has been reported. This particular fusion protein represents the start of the second ORF. It was unreactive with all BVDV antisera This may be due to the structure of the fusion-protein, epitopes normally displayed on the mature virus protein may become inaccessible when they form part of the fusion protein.

Alternatively, this particular protein may be poorly immunogenic in vivo. This seems unlikely since antisera raised against the fusion protein were clearly positive in ELISA, IP and IF tests using BVDV infected cells. Antisera against the fusion protein identified a 36K and 76K in IP and western blotting respectively. The 76K protein is the major band detected by IP using sera from infected animals. It is a non-structural protein as judged by its failure to co-purify with virus in sucrose gradients. None of the antisera raised against the fusion proteins was able to neutralise the virus.

In summary then, the first ORF may code for the structural proteins in the form of a large precursor which is then processed to produce mature polypeptides of 28K, 48K and 50K, the latter two being glycoproteins. Summing their molecular weights and adding an average 5K to each glycoprotein (to account for co-translational glycosylation) gives a size of

approximately 136K for the precursor. A protein of around this molecular weight has been detected in infected cells (Donis and Dubovi, 1987a).

It is proposed that the non-structural proteins are derived from the second ORF, which is larger than the first one. The largest protein so far detected in BVDV infected cells is 165K which presumably represents the non-structural precursor. The first part of this precursor appears to become a 76K non-structural protein, possibly involved in viral RNA replication.

1.5.3 Conclusions and comparisons

Although the sequences of four pestivirus are now known, virtually nothing is known of their replication strategies and control mechanisms. Presumably the non-structural proteins are involved in the synthesis of the minus template strand and in processing of protein precurors, but no such roles have yet been assigned. The timing and appearance of each protein is largely undefined and the regulatory processes controlling the expression of the viral genes are totally obscure.

However, recent work on amino acid sequence comparisons has revealed that the p125 protein of BVDV contains domains with motifs characteristic of both serine protease type enzymes and helicases (DNA unwinding proteins). Even more interestingly, the positioning of these domains on the p125 protein is such that after the processing to p54 and p80 which occurs in CP BVDV strains, both the protease and the helicase are present on the p80 region (Bazan & Fletterick 1989 ; Gorbalenya et al 1989).

In addition, the BVDV genomic RNA has been postulated, on the basis of circumstantial evidence, to have a complex and extensive secondary structure. This structure has not yet been predicted from the sequence data.

Another puzzle which may be solved by determination of further sequence data from different BVDV strains is whether or

not actual physical recombination can occur between pairs of persistently infecting and super-infecting viruses in vivo. This has been suggested to occur on the basis that some CP strains have been reported to be altered in their properties (both in pathogenesis and protein composition) following superinfection (Brownlie *et al*, 1989). Analysis at the nucleic acid level could define this very precisely - if this is occurring it has considerable epidemiological implications regarding the antigenic variation of these viruses.

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter covers all the basic techniques used throughout this work, but some more detailed methods will be found in the appropriate chapters.

2.1 TISSUE CULTURE

Cells: The cells used were semi-continuous cultures of foetal lamb muscle (FLM), bovine turbinate (BT) and embryonic bovine trachea (EBTR).

Cultures were established and propagated using standard techniques (Paul, 1970). Briefly, tissues were coarsely chopped then disaggregated using 0.25% trypsin, filtered through gauze and washed in tissue culture medium. Cells were seeded at 1ml pcv per 100ml medium, incubated for 24hrs, then the medium was changed. Cultures were allowed to reach confluence and were subsequently split 1:3 when necessary.

Cultures were used between pass 5 and pass 20, after which growth rate and susceptibility to virus infection were reduced. All cell stocks used were shown to be free of contaminating pestiviruses by an indirect immunofluorescence test (IFT). Cell cultures were also checked periodically every 5 passes by IFT, as described previously (Gardiner *et al*, 1983; Nettleton *et al*, 1987).

Tissue culture medium and solutions

1 x Hanks balanced salt solution (Hanks BSS)

	mg/l
$MgSO_4$; $7H_2O$	200
$MgCl_2$; $6H_2O$	100
$CaCl_2$; $2H_2O$	140
NaCl	8000
Na_2PO_4; $7H_2O$	3000
KH_2PO_4	60
KCl	400

1 x phosphate buffered saline

	mg/l
NaCl	8000
KCl	200
Na_2HPO_4	1150
KH_2PO_4	200

1 x isotonic saline

NaCl	8500

Medium E199

E199 10x liquid with Earles salts (modified), without sodium bicarbonate and L-glutamine (Flow Labs). Ix stock was prepared as follows:

Constituents	Stock concentration	Volume	Final concentration
E199	10x	50ml	1x
Tryptose phosphate broth	29.5g/l	60ml	3.55g/l
Sodium bicarbonate	80g/l	3ml	0.48g/l
Distilled water to 500 ml			
For use add:			
L-glutamine	0.1M	5ml	1mM
Penicillin	10,000u/ml	5ml	100u/ml
Streptomycin	10g/l	5ml	0.1g/l

* 10% serum for growth medium

2% serum for maintenance medium, a further 3ml sodium bicarbonate to give 0.9g/l.

* Serum used was Foetal Bovine Serum (FBS) from Northumbria Biologicals (batch 37826), shown to be free of BVD virus and antibodies to BVDV. The serum was heat inactivated for 30 min, 56°C.

In some later experiments horse serum (non heat-inactivated) from Tissue Culture Services Ltd (batch 480) replaced FBS.

IMDM

IMDM (powder) Iscoves modified Dulbecco's Medium, with L-glutamine, with 25mM Hepes buffer, without sodium bicarbonate, without alpha-thioglyceral, without beta-mercaptoethanol (Gibco Labs). Ix stock was prepared as specified in Gibco catalogue. For use pencillin (100,000u/ml) was added to a concentration of 100u/ml, streptomycin (10g/l) was added to 6.1g/l. Sera, as detailed above, were added to concentrations of 5% for growth medium and 2% for maintenance medium.

2.2 VIROLOGY

2.2.1 Virus strains

The two main pestivirus strains used throughout this work were the BDV strain G1480 (non-cytopathic) and the BVDV strain NADL (cytopathic). Occasionally other strains were used in specific experiments and these will be detailed in the appropriate chapter.

BDV-G1480 was isolated at Moredun from a precolostral blood sample taken from a new born lamb with clinical symptoms of Border Disease. The lamb's mother had been infected experimentally at 52 days gestation with the Moredun strain of BDV isolated originally from a pool of brain tissue (IIB brain pool) from newborn lambs clinically affected with BD (Barlow *et al*, 1980). It was passaged five times in foetal lamb kidney (FLK) cells including three passages at terminal dilution. This master stock was adjusted to 10^5 $TCID_{50}$/ml and stored in 20ml aliquots at -70°C.

BVDV-NADL was originally isolated from the spleen of an animal which died from BVD at the National Animal Diseases Lab, Iowa, USA. It was obtained from the American Type Culture Collection and kindly supplied by Dr. P.L. Roeder, Central Veterinary Laboratory, Weybridge. The virus had been passaged seven times in bovine cells and was passaged a further twice

then stored as a master stock of titre 4 x 10^6 $TCID_{50}$/ml in 4ml aliquots at -70°C.

Working stocks of both strains were grown as required.

2.2.2 Virus growth

Viruses were grown on 100% confluent cell monolayers. BDV was grown in FLM cells. BVDV was grown in bovine cells (BT or EBTR). Depending on requirements, virus was grown in plastic tissue culture flasks (various sizes) or in glass roller bottles surface area 1500cm^2 (Jencons). All cultures were sealed and incubation was in a non-CO_2 atmosphere at 37°C.

Confluent monolayers were washed twice with warm Hanks BSS. The virus inoculum was added at the appropriate multiplicity of infection (moi) generally 0.01 TCID50/cell and in a minimal volume, 20ml per roller bottle, for example. The cultures were incubated at 37°C for 1hr with occasional agitation, then the inoculum was removed and maintenance medium was added. Incubation of CP viruses was continued at 37°C for 2-5 days, depending on virus strain and degree of cytopathic effect (CPE) at harvest. NCP viruses were harvested after 4-5 days.

2.2.3 Virus harvesting

The sources of infectious virus were: the tissue culture supernatant and the cell monolayers. For BVD-NADL the distribution of virus has been shown to be approximately 10 fold higher in the supernatant than in the cellular fraction 12hrs post infection (pi) (Nuttall, 1980). For BD NCP virus the distribution is approximately equally divided between the two compartments (P. Nettleton, personal communication).

During this work, infectious virus was harvested from tissue culture supernatant only, and the cells were further processed to yield viral RNA.

Procedure: The tissue culture fluid was removed from the cultures and clarified by centrifuging at 9000rpm, 30 min at 4°C in a Beckman Ja-14 rotor for the Beckman J2-21 high speed centrifuge. This clarified supernatant was sampled for titration and aliquots stored as working stocks as required.

Supernatants not used immediately for virus isolation were stored at -70°C until required.

Various methods were tested to find the most efficient procedure for concentrating supernatants and purifying virus. These are discussed fully in Chapter 3, but the usual means of isolating intact virus from supernatant was to centrifuge the supernatant at 25K, 16hrs at 4°C in an SW28 rotor.

2.2.3 Assay of infectious virus

Tissue culture supernatants were assayed for either cytopathic or non-cytopathic virus by an end point titration method. Serial ten-fold dilutions of samples were made in serum-free medium and titrated in 96 well microtitre plates. Four wells each containing 25ul volumes were used per dilution. The appropriate host cells in 100ul of growth medium were added in the form of a suspension containing 2×10^4 cells per 100ul per well and the plates were sealed and incubated at 37°C for 5 days. The titration end point was determined for CP viruses by the microscopic examination of cultures for cytopathic effects. For NCP viruses the end point was determined by an immunoperoxidase staining procedure as detailed below. In both cases the titration end point results were calculated and expressed as Tissue Culture Infective Dose/ml ($TCID_{50}$/ml) (Lenette and Schmidt, 1979).

Immunoperoxidase test for detection of NCP virus (Smith *et al* 1988)

Following 5 days incubation, the plates were emptied, gently washed with warm physiological saline and air-dried. Cells were fixed to the plates in 95% acetone in distilled water at -20°C

for 30 min. The plates were then air dried, washed once more in saline and blotted dry.

Fifty microlitres of an optimal dilution of gnotobiotic lamb serum with a high level of neutralizing antibody to BDV were added to each well and incubated at 37°C in a moist atmosphere for 30 mins. Plates were then washed four times in warm wash fluid and blotted dry.

Fifty microlitres of the 2nd antibody: donkey anti sheep-horseradish peroxidase conjugate from the Scottish antibody production unit (SAPU) were added to each well and the plates incubated 30 min as before. The four washes in warm wash fluid were repeated and the plates blotted dry.

Freshly prepared substrate AEC (see Appendix 1) was added (50ul) to each well. Colour development occurred after 30 min - 1 hr at room temperature. The reaction was stopped by washing plates once in tap water.

2.2.4 Trichloroacetic acid (TCA) assay of incorporation of ^{3}H-uridine into cellular RNA

Cells (FLM) were grown on the inside of stoppered glass test tubes in E199 growth medium containing 2uCi/ml ^{3}H uridine. Medium was aspirated from the tubes and the monolayers washed twice with ice cold PBS. 0.5ml of lysis buffer was added to each tube (Lysis buffer: 0.5g NP40, 0.017g NaCl, 0.03g $MgCl_2$, 1ml Tris pH8.6, 100ml H_2O). Tubes were vortexed thoroughly for 10 seconds.

10ml ice-cold 5% TCA were added, mixed and the tubes incubated at 4°C for 5 min.

The contents were then filtered through a Whatman glass fibre filter using a vacuum pump attached to a conical flask with a funnel attachment.

The filter was washed twice in ice-cold TCA, once in absolute ethanol, air dried and placed in a scintillation vial. Counts were obtained over 1 minute in PPO scintillator fluid on a Scintillation Counter.

2.3 DNA TECHNIQUES

2.3.1 Standard solutions

All solutions were sterilised by autoclaving at 15lbs/inch^2 for 20 min unless otherwise indicated.

1 x TE:	10mM Tris HCl, 1mM EDTA adjusted to pH 8.0 with concentrated HCl.
1 x TNE:	100mM NaCl 10mM Tris HCl pH8.0 1mM EDTA pH8.0
50 x TAE:	2M Tris base, 1M CH_3COO Na, 50mM EDTA, stock solution adjusted to pH8.3 with glacial acetic acid.
20 x SSC:	3M NaCl, 0.3M sodium citrate, stock solution adjusted to pH7.0 with 5N NaOH.
50 x Denhardts solution:	1% (w/v) Ficoll, 1% (w/v) polyvinyl pyrrolidone, 1% (w/v) BSA (Pentax fraction V), filtered through 0.45um filter and stored -20^{o}C.
Ethidium bromide:	10mg/ml in H_2O stored in light-proof bottle.
Water:	double-distilled, deionised sterile water.

Organic Reagents

Phenol: Redistilled from solid. Distillate was equilibrated with 1M Tris-HCl pH8.0 and stored under a layer of TE buffer. 8-Hydroxyquinoline was added to 0.1% (w/v). Aliquots were stored at -20°C.

Chloroform: chloroform and isoamyl alcohol (IAA) 24:1 mixture stored at room temperature.

Phenol/Chloroform: 1:1 mixture of the above.

Formamide: Deionised by adding 1g/ml of ion exchange resin (Dowex AGx G8) and stirring for 1hr. The formamide was filtered through Whatman No. 1 paper and stored at -70°C in small aliquots.

Glassware and plasticware

All glassware used in nucleic acid work had been washed, rinsed twice in double-distilled water and sterilised by autoclaving (15lbs/in^2 20 min). Further treatment was necessary for glassware to be used in RNA work: see Section 4. Pipette tips were sterilised by autoclaving as above.

Bacterial growth media

L-broth: 10g/l Difco Bacto tryptone
4g/l Bacto yeast extract
10g/l NaCl
pH 7.2

L-agar: As above plus 15g/l Difco agar.

Antibiotics: Ampicillin (500x) stock solution, 25mg/ml in water. Use at 50ug/ml.
Chloramphenicol: (200 x) 34 mg/ml stock solution in 70% ethanol. Use at 170ug/ml.

2.3.2 Phenol extraction of DNA

Generally done in 1.5ml microfuge tubes. An equal volume of phenol/$CHCl_3$/IAA mixture was added to the DNA solution. The pH of the equilibration buffer was 8.0. The mixture was vortexed thoroughly for 5 sec and the phases separated by a brief 1-2 min spin in a microfuge. The upper aqueous phase containing the DNA was removed and retained. The organic phase was then re-extracted with an equal volume of TE buffer and this was pooled with the first aqueous phase.

2.3.3 Ethanol precipitation

DNA was precipitated from solution by the addition of 0.5 volume of 7.5M ammonium acetate and 2 volumes ethanol. The tubes were spun immediately for 20 min to pellet the DNA (12000rpm in a microfuge (MSE) or high speed centrifuge depending on the volumes). Occasionally, when the DNA was at a very low concentration (< 0.1ug/ml) and recovery was critical, the ethanol/nucleic acid mixture, was stored at -20°C for 30-60 min before pelleting. The pellets were washed with 70% ethanol, dried in a vacuum desiccator and resuspended in the desired volume of TE buffer. DNA was stored in TE at 4°C.

Restriction endonuclease digestion of DNA

DNA was digested in a total volume of 10-50ul. The reaction mixture contained DNA, 10 x restriction buffer, restriction enzyme and water. Incubation was at 37°C for 1-2hrs. The BRL REACT buffer system was used, along with the appropriate enzymes. Reactions were generally loaded directly onto an agarose gel for analysis without further purification.

2.3.4 Preparation of competent cells and transformation with plasmid DNA

The method used was based on that of Chung *et al* (1989).

The bacterial strain used was *E. coli* JM109, Genotype: (lac-pro), thi, supE44, recA1, and A1, gyr A96, hscl, R17 /F^1, tra D36, $proA^+B^+$, lac/^{9}, lacZ ΔM15

A fresh overnight culture was diluted 1/100 into L-broth and the cells incubated with shaking at 37°C (225rpm) until the OD_{600} = 0.3-0.4.

The cells were pelleted 10mins at 4000rpm 4°C, resuspended in 1/10 volume of ice cold TSS and mixed gently. 0.1ml of this suspension was then added to a chilled polypropylene tube containing 1ul (0.01-1ng) of plasmid DNA and mixed gently. The tube was incubated on ice for 30-60min. 0.9ml of L-broth with 10mM glucose was added and the cells incubated at 37°C with shaking (225rpm) for an hour, to allow expression of the antibiotic resistance gene. 0.1ml of the culture was plated directly by spreading on L-agar plates containing the appropriate selective antibiotic

TSS: L broth + 10% (w/v) PEG 8000
5% (v/v) Dimethylsulfoxide (DMSO)
20ml $MgCl_2$
pH 6.5

2.3.5 Large scale preparation of plasmid DNA

500ml of L-broth plus ampicillin (25ug/ml) were inoculated with 20ml of an overnight culture in a 2-litre flask, and incubated 16hr at 37°C with vigorous shaking.

The cells were pelleted at 4000rpm for 15min at 4°C and the culture medium allowed to drain from the pellets. The pellets were washed in ice cold TNE and pelleted as above.

The pellet was resuspended in 10ml Lysis buffer (25mM Glucose, 25mM Tris HCl pH8.0, 10mM EDTA) to which lysozyme had been freshly added to a concentration of 2mg/ml. The suspension was stored at room temperature for 5min., 20ml freshly prepared solution 2* were added and the contents mixed thoroughly by inverting the bottle. The mixture was allowed to stand at room temperature for 10min. 15ml of ice cold solution 3* were added and the contents mixed by vigorous shaking then stored on ice

for 10min. The lysate was then centrifuged at 10000rpm for 30 min and the supernatant filtered into a fresh bottle. 0.6 volumes of isopropyl alcohol were added, mixed well and left for 10min at room temperature.

The DNA was recovered by centrifugation at 12K for 30min at 20°C. The pellet was rinsed with 70% ethanol and dried by evaporation at room temperature. The pellet was dissolved in 8ml of TE.

* Solution 2: 0.2N NaOH, 1% SDS
  Solution 3: 60ml 5m KOAc, 11.5ml glacial acetic acid, 28.5ml H_2O

Purification of plasmid DNA on continuous CsCl density gradients

Eight grams of solid CsCl were added to the 8ml of DNA solution (final volume = 10ml). 0.8ml of a 10mg/ml ethidium bromide solution was added and mixed with the CsCl solution. The final density of the solution was 1.55g ml^{-1} (refractive index = 1.3860) and the ethidum bromide concentration was approximately 740ug/ml.

The mixture was transferred to a Beckman quick-seal polyallomer tube using a wide gauge needle and disposable syringe. The tube was filled with light paraffin oil and heat sealed. The density gradient was spun in a Ti 70 rotor (Beckman) for 48hrs at 40000rpm.

After centrifugation the gradient tube was viewed under UV light, to visualise the DNA bands. The lower, plasmid band was collected through the side of the tube using a syringe and a 19g needle.

The plasmid DNA was placed in a 10ml polypropylene tube and the ethidium bromide was removed by four extractions with an equal volume of n-butanol. The CsCl was removed by dialysing against several changes of TE buffer overnight. The concentration of plasmid DNA was measured by checking the

$OD_{260/280}$ of the solution and the DNA was precipitated by ethanol, resuspended to the desired concentration in TE, aliquoted and stored at -20°C.

2.4 RNA TECHNIQUES

2.4.1 Precautions to minimise RNAse activity

All glassware for RNA work was washed and sterilised as for normal laboratory glassware then soaked in a 0.1% (v/v) solution of diethyl pyrocarbonate (depc) in water overnight and sterilised by autoclaving. Where possible the use of sterile disposable plasticware was preferred, as this was assumed to be RNAse free.

All solutions for RNA work were prepared from separate bottles of dried chemicals, depc was added to 0.1% v/v and the solutions left overnight at room temperature then autoclaved. Tris solutions were prepared using sterile depc-treated water, because the addition of depc to Tris causes its decomposition.

Depc is unstable in the presence of water and rapidly decomposes to ethanol and CO_2. For this reason depc was purchased in small (25ml) bottles, stored at 4°C in a dessiccator jar, and any excess was discarded after 1 month.

All RNA work was carried out wearing gloves to avoid contamination with RNAases from the skin.

2.4.2 Solutions for working with RNA (prepared under RNAase free conditions).

RNA extraction buffer. 4M Guanidinium isothiocyanate, 0.1M Tris Cl pH 7.5

B-mercaptoethanol, 1%

5.7M CsCl in 10mM EDTA

TE/SDS 10mM Tris pH7.6
1mM EDTA
0.1% w/v SDS

TNE/SDS 10mM Tris pH7.6
1mM EDTA
100mM NaCl
0.1% w/v SDS

2M $LiCl_2$

2.4.3 Phenol extraction

Phenol was equilibrated with ACE buffer (10 mM NaOAC pH5.1, 50mM NaCl, 5mM EDTA) An equal volume of phenol was added to the RNA solution, mixed well and the phases separated by centrifugation at 10000rpm for 5min, or in a microfuge. The aqueous phase was re-extracted with a phenol/$CHCl_3$/IAA mixture as for DNA. Phenol extractions were carried out on ice unless SDS was present in the sample.

2.4.4 Ethanol precipitation

RNA was precipitated from solution by the addition of 0.1 volume of 3M sodium acetate and 3 volumes of ice cold ethanol. The mixture was chilled at -20°C or -70°C for a minimum of 30min. The precipitate was collected as for DNA precipitation. The pellets were rinsed in 70% ethanol and resuspended in TE/SDS.

Storage: Long term storage (> 1 month) of RNA was in 70% ethanol as a precipitate at -70°C. Short term storage was as either a precipitate or an aqueous solution containing 0.1% SDS at -20°C.

2.4.5 Isolation of RNA using guanidinium salts followed by CsCl gradient purification (Chirgwin *et al*, 1977).

From a confluent cell monolayer rinsed well with Hanks BSS: RNA extraction buffer was added directly to the monolayer in the flask and the cells scraped off with a rubber policeman. The viscous lysate was transferred to a tissue homogeniser tube and homogenised for 1min. The homogenate was centrifuged at 4000rpm for 5min at 4°C. It was then layered onto a cushion of 5.7M CsCl in a Beckman polyallomer tube. Depending on the volume, either SW55 or SW40 tubes were used. The CsCl cushion occupied a maximum of 0.25 volume of the tube. Centrifugation was at 30K for 24hrs (SW40) or 40K for 16hr (SW55) at 20°C, in a Beckman L8 ultracentrifuge.

Following centrifugation, the supernatant was carefully removed down to the level of the cushion. The upper portion of the tube was cut off using a red hot scalpel blade and the remaining fluid drained off. The pellet was rinsed with 70% ethanol, dissolved in TE/SDS and transferred to a microfuge tube for ethanol precipitation.

RNA was also isolated from frozen virus pellets in SW28 tubes by adding the RNA extraction buffer directly to the frozen pellet and continuing as above.

2.4.6 Rate zonal sedimentation of RNA through sucrose density gradients

For this work the gradients used were 10-30% continuous preformed sucrose gradients.

Gradient preparation: stock sucrose solution was 60% (w/w) prepared in TNE/SDS using RNAse-free highly pure sucrose (BRL). Dilutions from this stock were made to give 10% w/w and 30% w/w solutions. The dilution was non-linear, see below.

Desired final concentration	Volume of 60% stock sucrose diluted to 1 litre (ml)	Density (g/cm^3)
10%	134	1.0381
30%	437	1.1270

(Taken from Meth Enzymol, Vol. 45)

Gradients were poured light end first into Beckman SW40 polyallomer tubes, using a three channel peristaltic pump. All pump tubing, gradient tubes and apparatus had been soaked in 0.1% depc in water, then rinsed with depc treated water prior to use. Gradients were used within 30min of preparation.

The RNA samples were dissolved in 100ul TNE/SDS and heated to 65° for 15min prior to loading on the gradient.

Centrifugation of the gradients was performed at 20°C 20000rpm for 20hr, and the rotor decelerated without braking.

Following centrifugation, tubes were carefully removed and the gradient was fractionated by upward displacement using 40% sucrose solution pumped into the bottom of the tube. The gradient was collected in 0.5ml fractions while the OD was measured. The desired fractions were pooled and precipitated with ethanol. All fractionation equipment had been depc treated prior to use. The gradient profile was seen as a spectrophotometric trace.

2.5 GEL ELECTROPHORESIS

2.5.1 Horizontal agarose gel electrophoresis of DNA

Buffers: Running buffer: 1 x TAE Ethidium Bromide (EtBr) 100ug/ml
Sample loading buffer: 5 x TAE, 50% (v/v) glycerol
0.5% w/v xylene cyanol
0.5% w/v bromophenol blue

The optimum percentage of agarose in the gel was determined by the size range of the DNA to be separated, as recommended in Sambrook *et al* (1989).

The agarose was dissolved in running buffer to the desired concentration by heating in a microwave oven. After cooling to 50°C the solution was poured into a gel casting tray (11 x 14cm) (BRL). When set, the gel was placed in the gel tank (BRL mini gel apparatus) and covered with running buffer. The samples were mixed with 0.2 volumes of loading buffer and loaded into the wells. Gels were run at 60-80v for 1-2 hrs or 15v overnight.

2.5.2 Formaldehyde-agarose gel electrophoresis of RNA

All solutions and glassware were RNAase free as described in Section 2.4.1.

Buffers: 10 x MOPS/EDTA: 0.2M MOPS [3-(N-morpholino) propanesulfonic acid]
50mM sodium acetate
10mM EDTA

Running buffer: 1 x MOPS/EDTA

Sample buffer: 0.75ml deionised formamide
0.15ul x MOPS
0.24ml 37% formaldehyde
0.1ml H_2O
0.1ml glycerol
0.8ul 10% (w/v) bromophenol blue

Gels were prepared in running buffer as for DNA agarose gels, and allowed to cool to 50°C. Formaldehyde was added to a concentration of 0.66M and the gel was poured into an 11 x 14cm tray and allowed to set for 1hr in a fume hood.

The RNA samples were prepared for electrophoresis by precipitating with ethanol, redissolving in 1-2ul of 25mM EDTA/0.1% SDS and mixing with 10ul of sample buffer. After heating at 65°C for 15min, 1ul of 1mg/ml EtBr solution was added and the samples were loaded onto the gel. Electrophoresis was generally performed at 60v for 2-3hrs.

2.5.3 Alkaline agarose gel electrophoresis of DNA

Buffers: 1 x running buffer: 50mN NaOH
1mM EDTA pH8.0

6 x sample loading buffer: 300mN NaOH
6mM EDTA
18% Ficoll (type 400; Pharmacia)
0.15% bromocresol green
0.25% xylene cyanol

The desired amount of agarose was dissolved in water and allowed to cool to 60°C. NaOH was added to 50mM and EDTA to 1mM. The gel was poured and allowed to set as described in Section 2.5.1. It was placed in the electrophoresis tank and sufficient 1x running buffer added to cover the gel to a depth of 2mm.

The samples to be electrophoresed (in H_2O) were prepared by adding EDTA to 10mM followed by 0.2 volume of 6 x sample loading buffer. Electrophoresis was performed until the dye had migrated approximately 2/3 of the way into the gel.

The gel was soaked for 30min in 7% TCA then mounted on three layers of 3MM paper, covered in Saran wrap and dried in a commercial gel dryer (Biorad) without heat for 2 hours. The dried gel was then autoradiographed.

2.5.4 Visualisation and photography of nucleic acids in gels

Ethidium bromide was included in the gel and the running buffer for ordinary agarose gels, and in the sample for RNA gels.

Gels were viewed on a short wave UV transilluminator and photographed using a Polaroid camera, Type 667 film under a red/orange filter. Exposure time was generally 0.5sec at f4.5

2.5.5 Recovery of DNA from agarose gels

The desired bands were eluted from the gel and the DNA purified by one of two methods either by using 'Geneclean' according to the manufacturers instructions (Stratech Ltd) or by electroelution as described by Sambrook *et al* (1989).

2.6 NUCLEIC ACID HYBRIDISATION ON FILTERS

2.6.1 Preparation of RNA dot blot filters

Filters were nitrocellulose (Schleider and Schuell) or nylon (Hybond N, Amersham). Gloves were worn at all stages of handling filters. The filter was moistened for 15min in distilled water, transferred to 20 x SSC for 30min and then applied to a commercial blotting manifold (Hybridot or hybrislot).

The RNA sample was denatured in a volume of 100ul containing 7.5% formaldehyde and 6 x SSC in TE buffer, by heating to 65^{o} for 15min before chilling on ice.

The sample was then applied to the manifold under a gentle vacuum such that 100ul sample took approximately 5mins to blot. The filter was removed and air dried on 3MM (Whatman) paper for 30min. The RNA was fixed to nitrocellulose filters by baking under vacuum for 2hrs and to nylon filters by brief (2min) exposure to short wave UV light. Filters not used immediately were stored between sheets of 3MM paper wrapped in Saran Wrap.

2.6.2 Preparation of northern blot filters

RNA samples were run on a formaldehyde gel as described in Section 2.5.2. RNA markers (BRL RNA ladder) were included on the gel and the gel was photographed under UV directly without destaining. The gel was then soaked in 10 x SSC for 2 x 20min periods.

Filters used were as described in the previous section. The filter to be used was pre-wet in distilled water for 5min then transferred to 10 x SSC for 5min. It was applied to the gel and blotted by capillary action using 10 x SSC as the transfer buffer, essentially as described in Sambrook *et al* (1989). RNA transfer was allowed to take place over 16hrs. The fixing of RNA to the filter, and storage was as described in the previous section.

2.6.3 Preparation of DNA probes by random priming (Feinberg and Vogelstein, 1984).

This method allows the labelling of isolated DNA fragments to high specific activities. Linear double-stranded DNA purified from agarose gels as described in Section 2.5.5 was used as the template in the reaction. The optimum size of template is 100-300bp.

Using ^{32}P as label, the resulting probe is only stable for around 4 days, so it was impractical to prepare excessive amounts. The amount of template in the reaction was determined by the eventual amount of probe needed for the hybridisation reaction (see below)

Typical hybridisation reactions:

	Area	Hybridisation volume	Probe	Template*
Dot blot filter	$5cm^2$	1.5ul	30-75ng	20-50ng
Dot blot filter	$10cm^2$	3ul	60-100ng	50-100ng
Northern filter	$100cm^2$	25ul	500-1000ng	250-500ng

* Assumes the amount of labelled probe produced is double the amount of input template, though labelling double-stranded template effectively halves the specific activity of the available probe.

Reagents:
- 2M Hepes pH 6.6
- Solution 1: 1.25M Tris pH8.0; 0.125M NaCl; 500ul each dATP, dGTP and dTTP
- Random primers: 90 OD units/ml of random hexamers (Pharmacia)
- Oligo-labelling buffer (OLB): 2M Hepes (250): Random primers (150): solution 1(100)
- Bovine serum albumin (BSA) (10mg/ul)
- dCTP 2.5mM

The correct amount of template DNA in 10ul distilled water was boiled for 3min, the volume restored to 10ul, then chilled on ice. The following components were added: 4ul OLB, 2ul dCTP, 1ul BSA, 2ul ^{32}PdCTP and 5 units large fragments of *E. coli* polymerase. Between 10^6 and 10^7 of label were used per ml of hybridisation buffer.

The resulting 20ul reaction was incubated at 37°C until approximately 70% incorporation of label was achieved (generally 2-3hrs)

The probe was purified from unincorporated labelled dCTP by ethanol precipitation using ammonium acetate. It was resuspended in 500ul of TE buffer and used immediately in a hybridisation reaction or stored at -20°C. Between 10^6 and 10^7 cpm/ml of hybridisation fluid were used.

2.6.4 Preparation of RNA probes

^{32}P-labelled single-stranded RNA probes were prepared by run-off transcription from a T7 promoter. The insert to be transcribed was contained in the transcription vector pGEM4. Details of this vector are to be found in Chapter 5.

The template was plasmid DNA which had been linearised by digestion with a restriction enzyme at a site downstream from the T7 promoter. The reaction was carried out in a 20ul volume. All solutions and equipment were treated to minimise RNAse activity as described in Section 2.4.2.

The reaction was carried out in 40mM Tris-HCl pH8.0, 10mM $MgCl_2$ 10mM Dithiothreitol (DTT), 4mM spermidine, 10mM NaCl and 50ug/ml BSA. The template DNA was added to the above buffer along with ATP, GTP and CTP each to a final concentration of 0.5mM. Generally, 0.5ug of template was used. 20uCi of ^{32}P-UTP were added followed by 10 units T7 RNA polymerase (Pharmacia). The reaction was incubated at 37°C for 1hr. Remaining DNA was removed by diluting the reaction to 200ul and adding 10 units DNAse 1 (Pharmacia) then incubating a further 15 mins at 37°C.

The RNA probe was purified by phenol extraction and ethanol precipitation, then by chromatography on a 10cm x 1cm Sephadex G50 column followed by a further ethanol precipitation. The probe was redissolved in TE buffer containing 0.1% SDS and either used immediately or stored frozen at -20°C.

2.6.5 Hybridisation and washing procedures

Hybridisation fluid: 50% formamide
5 x Denharts solution
4 x SSC
0.1% SDS
100ug/ml denatured salmon sperm DNA

Filters prepared as described in Section 2.6.1 were pre-hybridised in the above fluid for a minimum of 4hrs at 42°C. This and the subsequent incubation with probe took place in minimal volumes in sealed vessels. Examples of typical hybridisation volumes are given below

Filter size	Volume hybridisation fluid	Hybridisation vessel
$5cm^2$	1ml	10ml polypropylene tube
$10cm^2$	4ml	50ml polypropylene tube
$100cm^2$	10ml	200ml screw top roller jar

Continuous movement of the buffer over the filter during pre-hybridisation and hybridisation was provided by incubating the tubes or jars on a roller apparatus (or rotary shaker for 10ml tubes).

After the pre-hybridisation the required amount of probe was added directly to the hybridisation fluid. Double-stranded DNA probes were first denatured by boiling. Incubation continued at 42°C for up to 16hrs. This was the standard hybridisation procedure, variations on this method are described in Chapter 5.

Following hybridisation the probe and hybridisation solution were removed and either stored for re-use or disposed of. The filter was washed as follows, with agitation.

2 x 5 min in 2 x SSC 0.1%SDS at room temperature
2 x 30min in 0.1 x SSC 0.1%SDS at 55-60°C

Again, variations on these standard washes are described in Chapter 5.

The washed filters were wrapped in Saran Wrap and autoradiographed. They were not allowed to dry if they were to be re-probed. Probe could be stripped by washing 2 x 30min in distilled water at 70°C.

2.6.6 Autoradiography

Detection of ^{32}P radiolabelled material was performed using X-ray film in cassettes. Gels were dried and covered in Saran Wrap, wet filters were sealed in Saran Wrap. The package was placed in direct contact with the X-ray film and exposed at -70°C using a single intensifying screen on the opposite side of the film to the gel or filter.

2.7 POLYMERASE CHAIN REACTION

Both DNA and RNA templates were used in the Polymerase Chain Reaction (PCR) to amplify sequences. The specific protocols used are detailed in Chapter 6. The amplifications were performed using the Techne PHC-1 Dri block thermocycler and "Amplitaq" Taq polymerase enzyme (Perkin Elmer Cetus). RNA templates were converted to single strand cDNA using murine moloney leukaemia virus (MMLV) reverse transcriptase (GIBCO/BRL) prior to amplification.

PCR amplification buffer (10X): 500mM KCl
100mM Tris pH8.3
25mM $MgCl_2$
2mg/ml gelatin

Primers: Custom-made synthetic oligonucleotides used as PCR primers were obtained as aqueous solutions from Oswell DNA Service, University of Edinburgh.

2.7.1 Precautions to minimise contamination by irrelevant DNA sequences

All solutions and equipment were sterile and reserved for PCR work. Stock solutions were prepared using pipettes reserved for that purpose, aliquoted under sterile conditions and stored at -20°C. Different pipettes were used to handle post-amplification reactions and these reactions were never handled at the same time as pre-amplification reactions.

2.8 COMPUTING TECHNIQUES

The University of Wisconsin Genetics Computer Group (UWGCG) sequence analysis software packages, mounted on a VAX/VMS operating system were used. Details of the particular programs used are described in Chapter 7. The EMBL/GENBANK sequence data libraries were also accessed.

CHAPTER 3

GROWTH AND REPLICATION OF BORDER DISEASE VIRUS

3.1 INTRODUCTION

Large amounts of purified BDV were a necessary pre-requisite for this project. Before embarking on large-scale virus cultivation, however, studies were performed on the kinetics of virus growth and replication in order to optimise the amount of virus obtainable. This chapter first describes briefly previous studies on pestivirus growth, then presents and discusses results in which viral infectivity and RNA synthesis were measured over a period of time.

Previous reports have discussed the growth cycles of all three pestivirus species (Aynaud, 1968; Nuttall, 1978, 1980; Nettleton, 1985).

The growth curves of a cytopathic (CP) strain of BDV were compared to those of two CP BVDV strains in foetal lamb brain cells (Nettleton, 1985). No significant differences between strains were detected. Growth was exponential until about 20hrs post infection (pi) followed by a plateau.

Nuttall (1978, 1980) reported comparative growth studies (assaying infectious virus) between CP and NCP strains of BVDV. She concluded that although the rate of appearance of infectious virus was the same for both strains, the final yield was less (about 50 times) for the NCP strain. The effect of the drug Actinomycin D (AcD) on BVDV replication was studied. Virus infectivity was measured at 20hrs post-infection (pi) in the presence of 0.5-2.0ug/ml AcD for both CP and NCP strains of BVDV. Replication of the NCP strain was enhanced by 20-100%. Whether this effect was maintained throughout the growth cycle was not reported. Aynaud (1968) observed a similar enhancement of NCP HCV replication during the exponential phase in AcD-treated cells but this was a transient effect and a final

final reduction in virus yield was observed after treatment with AcD.

Reports on the kinetics of RNA synthesis of the pestiviruses are few. Nuttall (1978) followed the synthesis of BVDV RNA by incorporation of ^{3}H-labelled uridine. An estimate of viral RNA synthesis was obtained indirectly by comparing results from infected and mock-infected cells. However, this would not have discriminated between authentic viral RNA synthesis and host cell transcriptase responses, either positive or negative to viral infection. Viral RNA synthesis peaked at 10-11hrs pi, then incorporation fell rapidly. Labelled RNA could not be detected in progeny virions, suggesting that early RNA synthesis was not producing mature genome copies for packaging. In another study, (Karrar, 1983) the RNA synthesis of CP and NCP strains of BVDV was compared. Synthesis peaked after 12-14hrs, and CP strains showed a significantly higher final level of RNA synthesis, though the rates were similar. Again this author did not report the detection of the resultant labelled RNA in progeny virions.

Experimental

The experiments described fall into two sections, the first is a study of virus growth as measured by infectious virus released, and the second describes the measurement of RNA synthesis during virus infection by incorporation of tritiated (^{3}H) uridine into RNA.

Initially experiments were performed to determine the effect of the drug AcD on BDV growth. If there was enhancement of the growth of the NCP strain as described for BVDV (see above) then this would be useful to increase virus yields during large scale growth.

3.2 ACTINOMYCIN D AND VIRUS GROWTH

Methods

Virus growth curves were obtained under various conditions of treatment with AcD, to determine the optimum time of treatment

relative to infection, length of treatment and concentration of AcD to be used.

Cultures of FLM cells in $25cm^2$ flasks were infected with virus as described in Chapter 2, Section 2.2. The culture supernatant was sampled every 8 hours over a 72hr period, and the samples were assayed for extracellular virus titre by an immunoperoxidase test.

Actinomycin D was prepared as a 1mg/ml stock solution in PBS, filter sterilised and stored at -20^0C in the dark. Appropriate amounts of this stock were added directly to virus growth medium as required.

3.2.1 Results

Time-course of production of infectious BDNCP virus at various AcD concentrations

Duplicate 10ml cultures were set up and sampled as described above. AcD was added to the maintenance medium at the concentrations shown and was present throughout the 72hr period. The results of titration at different times are shown in Table 3.1 and Fig 3.1 shows a plot of the results at 0.01ug/ml AcD.

The presence of AcD does not appear to have any significant effect on virus growth at concentrations less than 0.01ug/ml. At 0.01ug/ml the drug enhanced the rate of virus production during the exponential phase, but this effect was transient, and an overall reduction in final titre by a log was observed.

Comparison of the effects of AcD on CP and NCP BDV strains

10ml cultures were set up for both cytopathic (BDV-M) and non-cytopathic (G1480) BDV strains. AcD was included in the maintenance medium at 0.01ug/ml and 0.05ug/ml. The control flasks had maintenance medium without AcD. All cultures were set up and sampled in duplicate. Sampling was every 8hrs for 72hrs and the extracellular virus was assayed as described in the previous experiment. Results are presented in Table 3.2 and Figure 3.2.

Table 3.1 Growth of BDNCP virus over 72hrs at various AcD concentrations

	AcD concentrations (ug/ml)			
Time (hrs)	0	0.001	0.005	0.01
8	2.6	2.6	2.6	2.6
16	2.8	3.3	3.3	3.8
24	3.1	3.8	3.3	4.8
32	4.1	4.3	4.3	5.6
40	5.1	5.3	5.3	5.8
48	6.3	6.3	5.8	5.6
56	6.3	6.3	6.0	5.6
64	6.3	6.6	6.0	5.6
72	6.8	6.3	6.0	5.2

Values are $\log_{10}$ of the mean $TCID_{50}$ measurement of duplicate cultures

Figure 3.1 Growth of BDNCP virus over 72hrs in the presence and absence of Actinomycin D (AcD). AcD was at 0.01ug/ml.

BDV growth in the presence of AcD

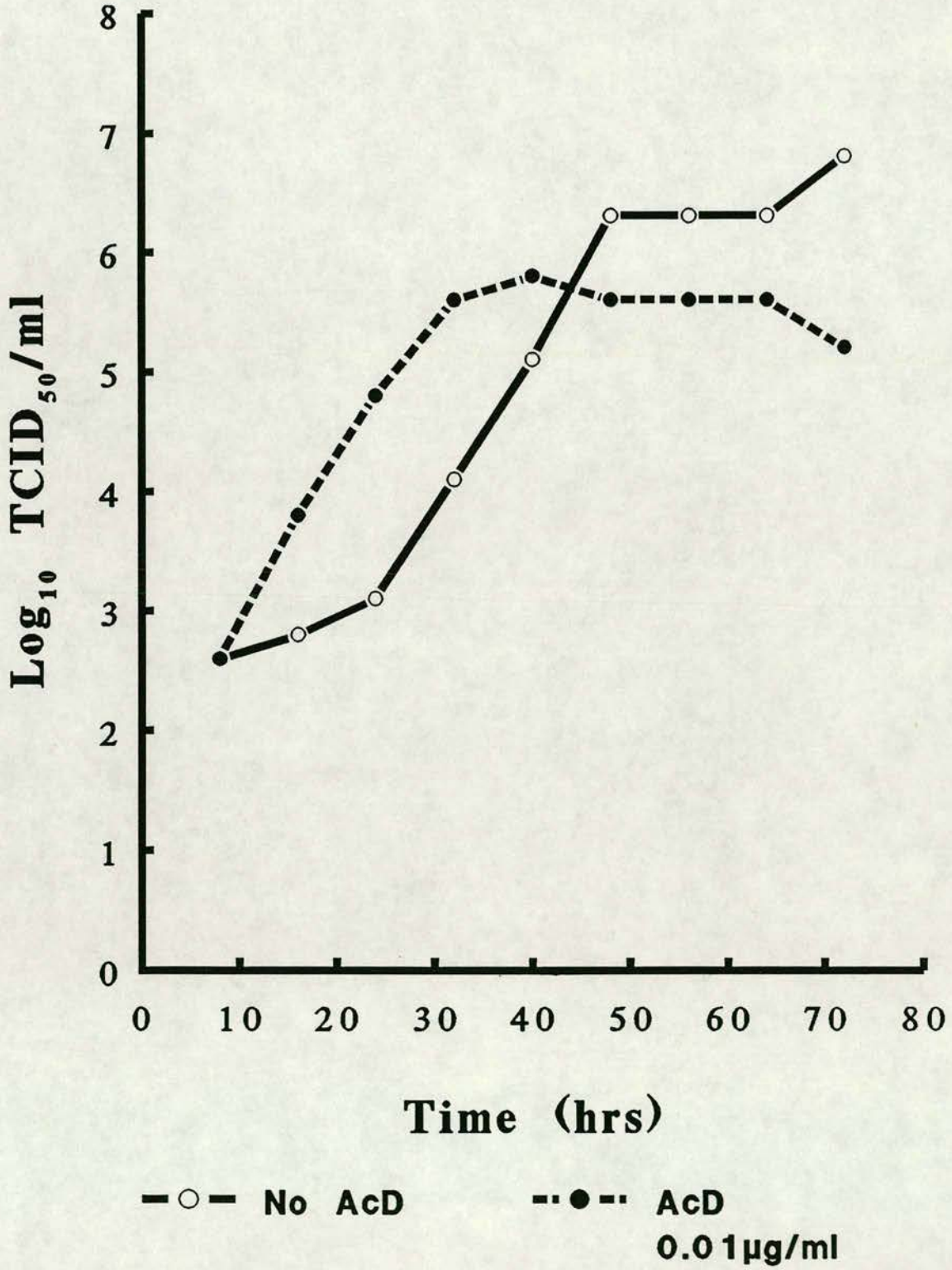


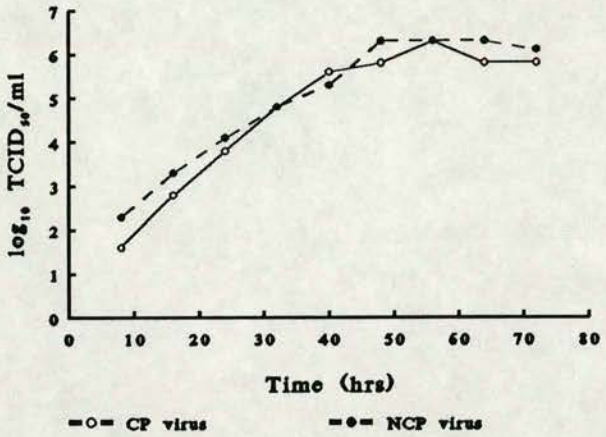
Table 3.2 Growth of CP and NCP strains of BDV in the presence of AcD

	AcD Concentration (ug/ml)					
	0		0.01		0.05	
Time (hrs) Virus type:	CP	NCP	CP	NCP	CP	NCP
8	1.6	2.3	2.6	2.6	2.3	2.6
16	2.8	3.3	4.3	3.8	3.8	4.3
24	3.8	4.1	4.8	4.8	4.8	4.8
32	4.8	4.8	5.3	5.8	5.6	5.8
40	5.6	5.3	5.3	5.8	5.6	5.8
48	6.3	6.3	5.3	5.6	5.3	5.2
56	5.8	6.3	5.3	5.6	4.6	5.2
64	5.3	6.3	5.1	5.2	4.2	4.6
72	5.8	6.1	4.8	5.2	4.2	4.6

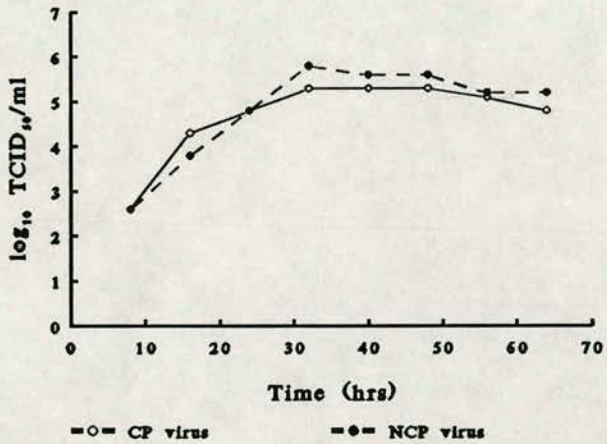
Values are $\log_{10}$ of mean $TCID_{50}$ measurements from duplicate cultures

Figure 3.2 Growth of cytopathic (CP) and non cytopathic (NCP) stains of BDV at different AcD concentrations. The top graph represents normal growth of both strains in the absence of AcD.

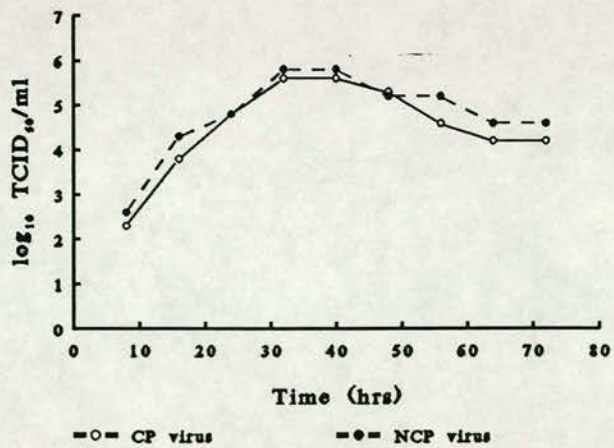
Effect of AcD on CP and NCP BDV growth



AcD 0.01ug/ml



AcD 0.05ug/ml



The growth curves of CP and NCP strains of BDV are not significantly different from each other in the absence of AcD, or in the presence of AcD at 0.01ug/ml. At 0.05ug/ml the drug appears to allow slightly better replication of the NCP strain throughout the growth cycle. As in the previous experiment AcD reduced final titres of virus for both strains, this effect increased at the higher (0.05ug/ml) AcD concentration. Thus both CP and NCP strains of BDV show similar growth responses to AcD; the replication rate is enhanced during exponential phase, the peak titre is unaffected but the final titre is reduced by 0.5-1 log. The initial enhancement of growth was the same for the two concentrations of AcD tested, but the final reduction was greater at the higher concentration. This is in contrast to the results reported for BVDV where the responses of the CP and NCP strains to AcD were completely different (Nuttall, 1980).

Effect of time of addition of AcD on the growth of BD NCP virus

In these experiments the effects of adding AcD to the cultures pre- and post-infection were examined.

Firstly, the effect of pre-treating host cells with AcD was examined. Cultures of FLM cells were pre-treated with AcD for 1 and 5hrs at 0.01 and 0.05ug/ml. Monolayers were washed thoroughly, infected with BDNCP as described previously then maintenance medium lacking AcD was added and sampled every 8hrs. All cultures were set up and sampled in duplicate. The results are shown in Table 3.3 and Figure 3.3.

As can be seen, pre-treatment of cells with AcD at 0.01ug/ml prior to infection resulted in an increased initial rate of virus production, reaching similar titres as untreated cultures. However, unlike the previous two experiments this time no final reduction in titre was observed after AcD treatment.

The rate of enhancement was similar whether cells were treated for 1 or 5hrs. Therefore 0.01ug/ml for 1hr was sufficient to achieve the effect.

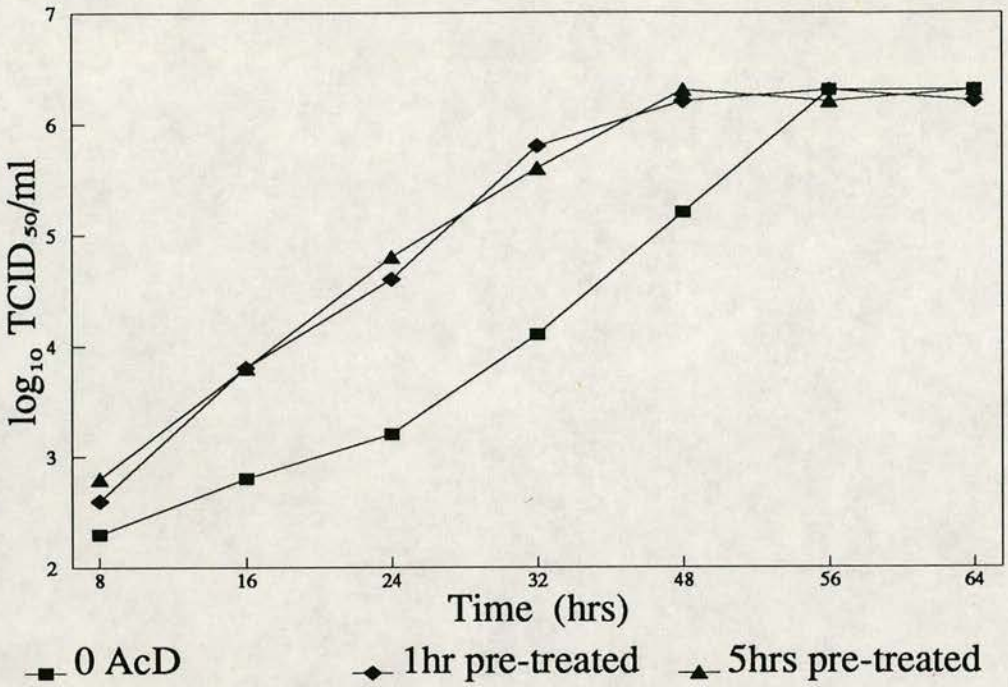
Table 3.3 Growth of BDNCP in cultures pretreated with AcD

		Concentrations of AcD ug/ml				
		0.01		0	0.05	
Time (hrs)	Length of Pretreatment	1hr	5hr	0	1hr	5hr
8		2.6	2.8	2.3	2.8	2.8
16		3.8	3.8	2.8	4.1	4.2
24		4.6	4.8	3.2	5.3	5.1
32		5.8	5.6	4.1	5.8	5.6
48		6.2	6.3	5.2	6.3	6.3
56		6.3	6.2	6.3	6.1	6.2
64		6.2	6.3	6.3	6.3	6.3

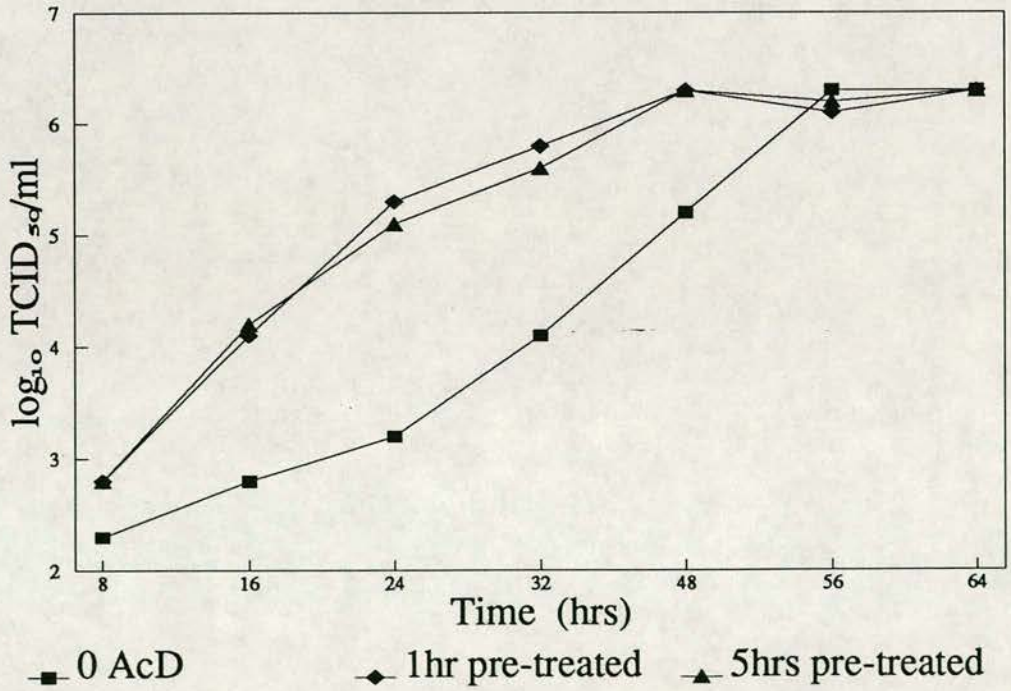
Values are $\log_{10}$ of the mean $TCID_{50}$ measurements from duplicate cultures

Figure 3.3 The effect of pre-treating host cells with AcD prior to virus infection.

0.01ug/ml AcD



0.05ug/ml AcD



Addition of AcD to virus infected cultures

AcD was added to virus infected cultures 12hrs after infection, at concentrations of 0.01, 0.05 and 0.5ug/ml (Figure 3.4, Table 3.4).

An interesting effect was observed at the lower concentrations - a very high rate of initial virus production was observed after AcD addition, this appeared to be concentration dependent. Cultures containing 0.01ug/ml AcD continued to produce virus until the final titre was equal to that of untreated cultures. Cultures containing 0.05ug/ml, although having a higher initial rate of virus production, showed a final virus titre of more than 1 log less than the control. This was perhaps due to a toxic effect of the drug on the cells.

3.2.2 Conclusions

Actinomycin D was shown to increase the rate of virus production during the early stages of infection with CP or NCP strains of BDV. However, there was also a concomitant lowering of the final titre achievable, unless AcD treatment was given for a short period pre-infection, or else 12hrs after infection. In no case did AcD increase the final titre of infectious virus over that of the untreated control cultures.

Thus AcD did not appear to be of use for increasing virus yield during large-scale virus production.

Table 3.4 Effect of addition of AcD 12hrs after infection with BDNCP

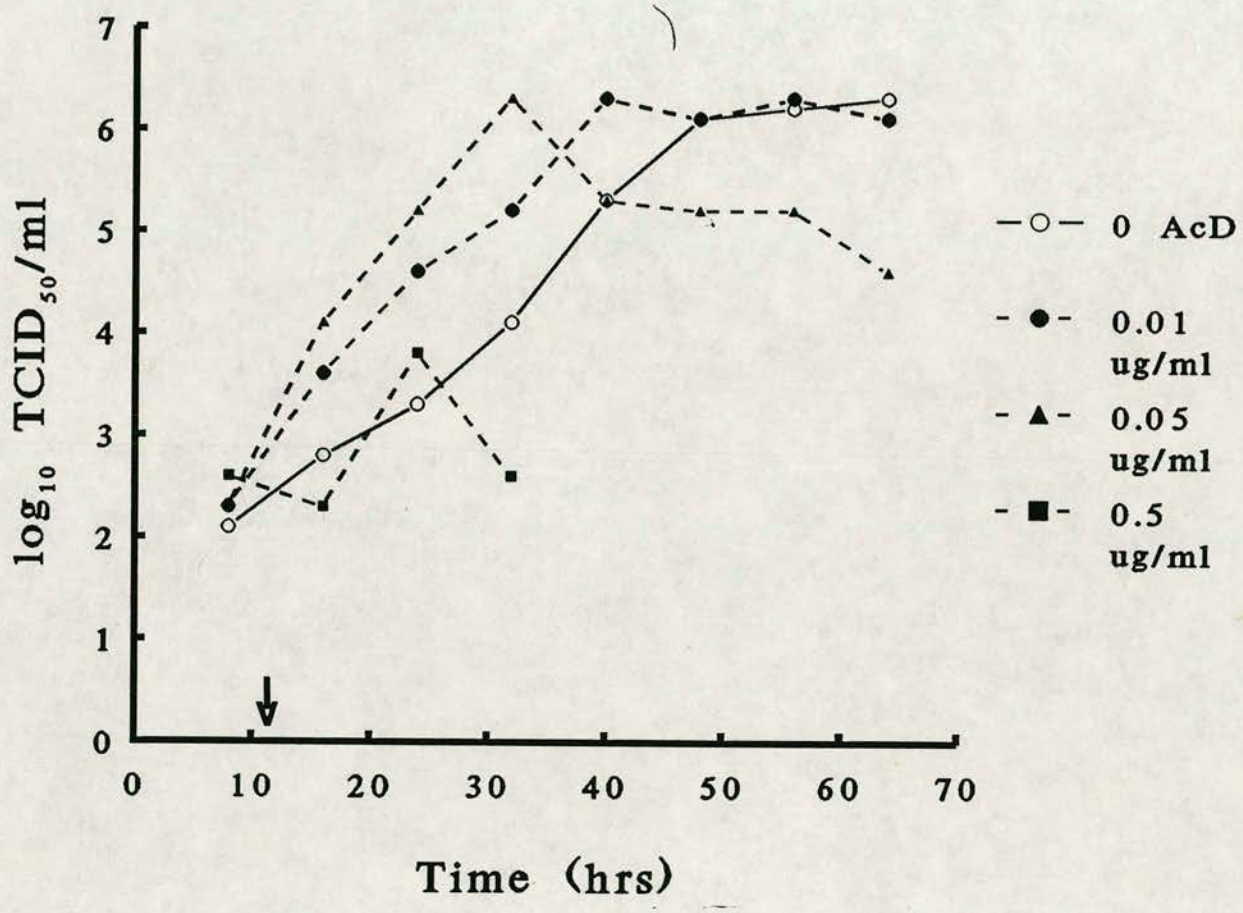
	AcD concentration (ug/ml)			
Time (hrs)	0	0.01	0.05	0.5
8	2.1	2.3	2.3	2.6
16	2.8	3.6	4.1	2.3
24	3.3	4.6	5.2	3.8
32	4.1	5.2	6.3	2.6*
48	5.3	6.3	5.3	0*
56	6.1	6.1	5.2	0*
64	6.2	6.3	5.2	0*

Values are $\log_{10}$ of mean $TCID_{50}$ measurements from duplicate cultures.

* Cells in these cultures had died at the time of measurement, presumably due to toxic AcD effects

Figure 3.4 Effect on BDNCP virus growth of addition of AcD to cultures 12hrs after infection. AcD was added where indicated by the arrow.

Addition of AcD 12hrs post infection



3.3 VIRUS REPLICATION IN THE INFECTED CELL

The aim of these experiments was to quantitate the kinetics of synthesis of BDNCP RNA during infection, and to provide information on the best time to harvest viral RNA from infected cells.

3.3.1 Experimental

The approach was to monitor the incorporation of the labelled precursor 3H-uridine into RNA during viral infection of cells. Actinomycin D was used to minimise incorporation of label into host cell RNA. By comparing incorporation of label in infected and uninfected cells an indirect measure of the amount of viral or virus-induced, RNA synthesis could be obtained. Conditions had to be established such that the availability of the labelled precursor was not rate-limiting in order that the amount of incorporation directly reflected the amount of RNA synthesis.

Potential reasons for label being rate-limiting were:
(a) the label is not available inside the cell, because the intracellular pool of uridine is not yet labelled to equilibrium. This can be prevented by pre-labelling the cells for the time it takes for the intracellular labelled and unlabelled uridine pool to reach equilibrium.

(b) The label is available intracellularly but is preferentially incorporated into host cell RNA. This can be prevented or minimised by treatment with AcD. The time of addition of label relative to AcD treatment also needed to be optimised.

3.3.2 Results

Determination of the time taken to label the intracellular uridine pool to equilibrium

15 tube cultures of FLM monolayers were incubated at 37°C (static) in E199 maintenance medium containing ^{3}H-uridine at 2uCi/ml. At the times indicated cultures were harvested in triplicate and assayed for the incorporation of the label into

TCA-insoluble material as described (Chapter 2, Section 2.6). Results are shown in Figure 3.5. Points are the mean of triplicate measurements.

The incorporation rate slowed after 1-2hrs, suggesting that the intracellular labelled and unlabelled uridine concentration is at equilibrium from this time. Pre-labelling the cells for 1hr should therefore be sufficient to ensure a constant intracellular concentration of label.

Determination of the timing and extent of inhibition of host RNA synthesis by AcD

Uninfected monolayers of FLM cells were incubated in the presence of ^{3}H-Uridine and AcD at various concentrations over a 24hr period. Cultures were harvested in duplicate at the times shown and assayed for ^{3}H-uridine incorporation. The results were expressed as % reduction in incorporation as compared with an untreated control culture. The results are shown in Figure 3.6.

At all AcD concentrations, the effect is at its peak 2hrs after treatment. Complete suppression of host cell RNA synthesis was not achieved in this or subsequent experiments. The best that could be achieved was 75-80% suppression after 24 hrs treatment with AcD at 1ug/ml. From the data presented in Figure 3.6 the optimum AcD treatment was deemed to be 0.1ug/ml for 2hrs, which gave around 70% suppression of host cell RNA synthesis.

^{3}H uridine incorporation in BDNCP infected, AcD-treated cells

2ml tube cultures were infected with BDNCP and the inoculum washed off after 1hr. Cells were then incubated in maintenance medium containing AcD at 1ug/ml for 2hrs followed by addition of ^{3}H uridine for 2hrs. Thus measurement of ^{3}H uridine incorporation could only be made from 4hrs post infection.

Initially cultures were assayed (in duplicate) 18hrs after infection and compared with uninfected cultures treated in the same way. The results are shown over.

Figure 3.5 Determination of the time taken to label the intracellular uridine pool to equilibrium using ^{3}H-uridine (2uCi/ml).

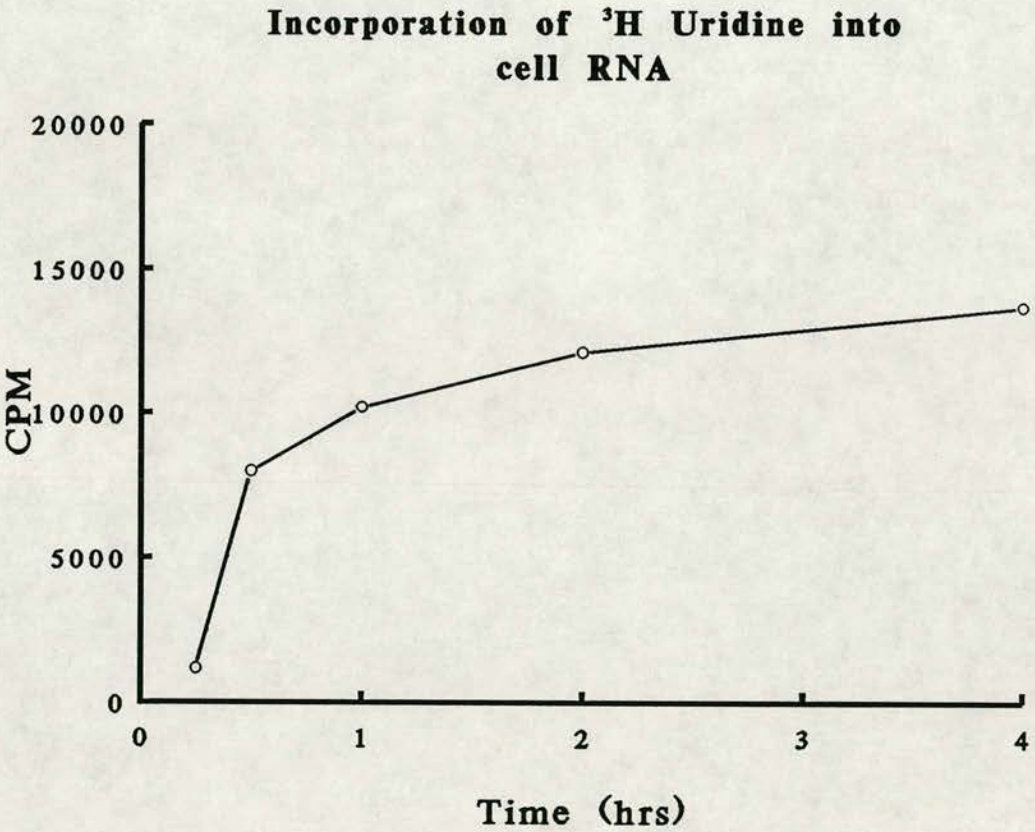
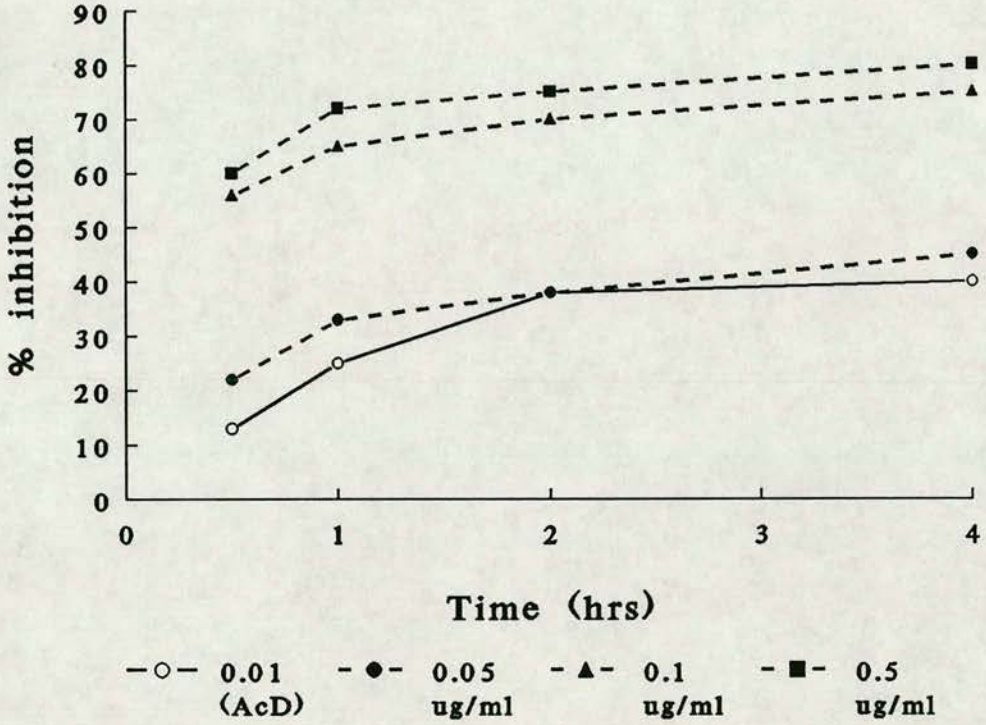

Incorporation of ³H Uridine into cell RNA
20000
15000
10000
5000
0
CPM
0
1
2
3
4
Time (hrs)

Figure 3.6 Time taken for AcD to exert an effect on RNA synthesis of uninfected FLM cells. Results are expressed as % reduction in ^{3}H uridine incorporation as compared to an untreated control culture.

RNA synthesis in AcD treated cells



Culture	Mean CPM/culture
No AcD uninfected	12723
AcD treated uninfected	213
AcD treated BDNCP infected	175

Thus there was no viral RNA synthesis detected after 18hrs, possibly because the labelled RNA had all been incorporated into progeny virions and released into the culture supernatant. The supernatant was not assayed for this. The next experiment assayed ^{3}H uridine incorporation in infected cells over a period of 20hrs starting from 4hrs pi as described above.

Time course of ^{3}H uridine incorporation in BDNCP infected cells

Cultures were infected and treated with AcD as described in the previous experiment. Duplicate cultures were assayed for ^{3}H uridine incorporation at the times indicated. Results are shown in Table 3.5, Figure 3.7.

Thus viral RNA synthesis is already under way by 4.5hrs pi, and rises rapidly to peak about 12hrs pi. The sharp decline which follows could either be due to release of this labelled fraction as whole virions, or as toxic effects of AcD on the cells. A similar sharp fall in RNA synthesis has been reported for BVDV (Nuttall, 1978).

3.3.5 Attempts to detect labelled genomic RNA in virions

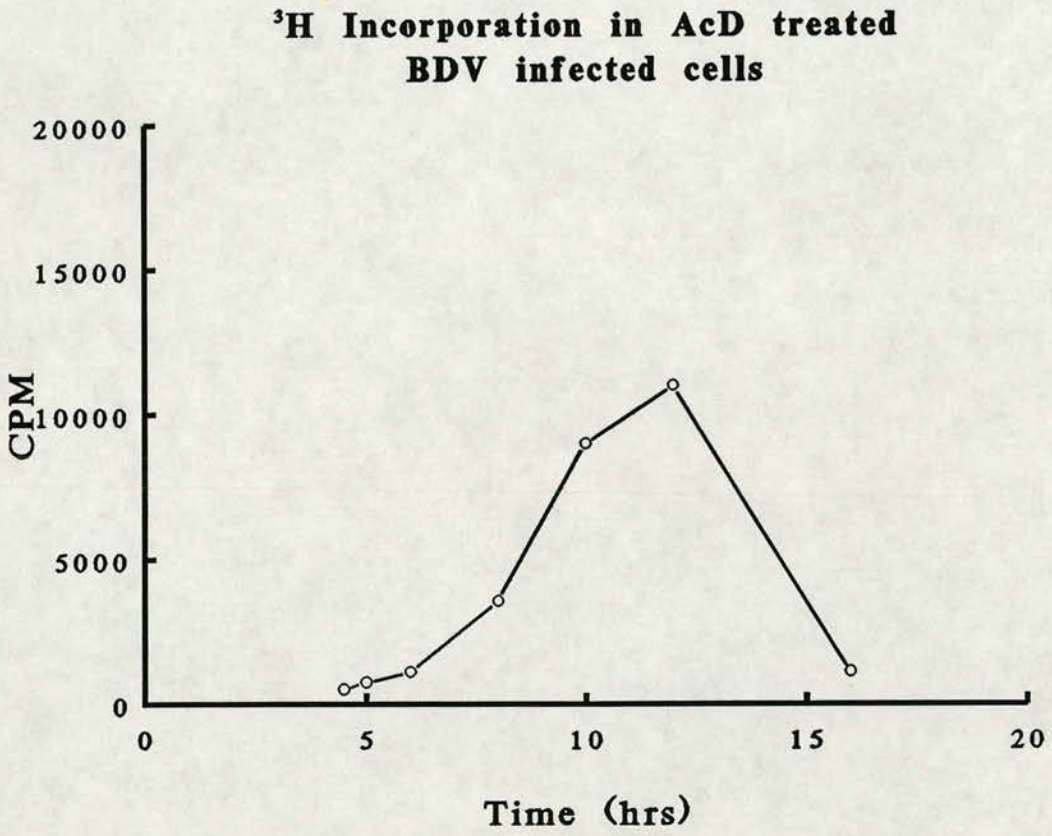
Using ^{3}H uridine as a precursor, attempts were made to label directly the virus genomic RNA, then purify the labelled RNA for analysis. The experiment was set up as follows:

Cultures of FLM monolayers in $100cm^{2}$ flasks were infected with BDNCP at an moi of 1.0 $TCID_{50}$/cell for 1hr. They were then incubated in medium containing AcD at 1ug/ml for 2hrs, then 0.1mCi of ^{3}H uridine was added to each culture and the cultures incubated for 24hrs. The medium was collected and clarified by centrifugation at 9K for 30min in a Beckman Ja14 rotor. Virus

Table 3.5

Culture	Time (hrs) 4.5	5	6	8	10	12	16
No AcD Uninfected	11175	11785	12098	12239	11462	101073	
lug/ml AcD Uninfected	386	307	319	294	342	-	-
lug/ml AcD BDNCP-infected	562	783	1131	3423	8936	11521	903

Figure 3.7 RNA synthesis in BDNCP-infected, AcD-treated cells as measured by ^{3}H uridine incorporation. Points are the mean of triplicate results.


^{3}H Incorporation in AcD treated
BDV infected cells
20000
15000
10000
5000
0
CPM
0
5
10
15
20
Time (hrs)

was pelleted from the supernatant at 25K for 16hrs in a Beckman sw28 rotor. The (invisible) pellets were resuspended in TNE and assayed for TCA insoluble counts and infectivity. The results are shown in Table 3.6. Clearly there is no labelled RNA present in the virus fraction. A second experiment produced similar results, ie infectious virus was recovered from the supernatant but it did not contain labelled RNA.

This failure to detect labelled RNA in the virus fraction was also encountered in experiments with BVDV (Nuttall, 1978). The suggested explanation was that the label was incorporated into a template RNA fraction on which the genomic RNA would later be transcribed. The template RNA would then be degraded. This would explain the sudden disappearance of labelled RNA in infected cells after 12hrs, in the previous experiment.

Thus attempts to identify and analyse the BDV genomic RNA by *in vivo* labelling using ^{3}H uridine were not successful. The next chapter discusses other approaches to the analysis of this RNA including further *in vivo* labelling attempts using ^{32}P orthophosphate.

3.4 LARGE SCALE PRODUCTION OF BDV

3.4.1 Introduction

The amount of BDV required for this work was estimated on the basis of a report of BVDV cloning (Renard *et al*, 1985). This report described the purification of 5ug viral RNA from 3 litres of tissue culture supernatant containing virus at 10^8 $TCID_{50}$/ml. Thus 3 x 10^{11} $TCID_{50}$ yielded only 5ug purified viral RNA. A major problem encountered with pestiviruses is that they grow poorly in tissue culture. This is especially true of BDV which, at the time of these experiments, grew to a maximum titre of only 10^6 $TCID_{50}$/ml. By the criteria above, we would need 3001 to produce 5ug BDV genomic RNA. A practical figure of 20-301 was decided as the target amount of supernatant to be produced. This should yield up to 0.5ug of viral RNA assuming a titre of 10^6 $TCID_{50}$/ml.

Table 3.6 Assay of extracellular virus for ^{3}H labelled RNA

Fraction	Titre $TCID_{50}$/ml From unlabelled control culture	cpm/ml From 3H labelled culture
Supernatant prepelleting (20ml)	2×10^{5}	2×10^{6}
Virus pellet (1ml)	3×10^{6}	437

The strategy was to have BDV in continuous production in glass roller bottle cultures of FLM cells, over a period of 30 weeks producing 1 litre of virus supernatant per week. The supernatant was clarified (9000rpm in Beckman Ja-14 rotor for 30min) and titrated then stored at -70°C until the total amount of supernatant had been amassed. The supernatants were then concentrated to a manageable volume, and virus spun directly out of suspension (25k 16hrs in Beckman sw28 rotor). Experiments described in the next section detail the design of the concentration and purification steps.

3.4.2 Experimental

FLM cells were grown to 100% confluence in "Jencons" glass roller bottles and infected with BDNCP G1480 master stock as described in Chapter 2, Section 2.2. The inoculum was left on the cells, with rolling for 1hr, then removed. 200ml maintenance medium were added and the cultures incubated with rolling for 5 days. Supernatants were clarified at 9K 30min and a 5ml sample removed for titration by immunoperoxidase staining. 100ml of one culture was stored as inoculum at -70°C. Table 3.7 shows results over a six month period of virus production, this was approximately half of the final amount of virus. There were many problems encountered at this stage and the pooled titre of the supernatants averaged 10^5 $TCID_{50}$/ml, over a log down on the optimum attainable. From this table there was a total of 6×10^9 $TCID_{50}$ of virus after 6 months. Virus production continued until 1×10^{10} $TCID_{50}$ had been obtained, in a total volume of 25 litres.

Table 3.7 Batch titrations of BDNCP virus during bulk growth

Batch date	Volume (ml)	Titre (log_{10} $TCID_{50}$/ml)	Total virus
24/02/88	200	2×10^4	4×10^6
2/03/88	400	2×10^5	8×10^7
8/03/88	100	4×10^5	4×10^7
9/03/88	2000	4×10^3	8×10^6
17/3/88	1000	7×10^4	7×10^7
17/3/88	500	1×10^6	5×10^8
30/3/88	500	7×10^5	3.5×10^8
18/4/88	2000	2×10^4	4×10^7
18/4/88	500	4×10^5	2×10^8
3/5/88	500	2×10^6	1×10^9
25/5/88	1500	1×10^5	1×10^8
7/06/88	500	1×10^6	5×10^8
12/06/88	500	1×10^5	5×10^7
17/06/88	1500	7×10^5	1×10^9
28/06/88	400	7×10^5	3×10^8
4/07/88	1500	1×10^6	1.5×10^9
12/07/88	800	1×10^4	8×10^6
2/8/88	800	4×10^4	3×10^7

3.4.3 Concentration and purification of BDV. Comparison of methods

Three methods of virus concentration were evaluated on a small scale to determine the most suitable method of concentration of the 25l of tissue culture supernatant. The methods compared were concentration by ultrafiltration, polyethylene glycol (PEG) precipitation, and ammonium sulphate precipitation. Three litres of BDNCP supernatant were pooled, clarified and split into 3 x 1000ml, 1000ml per method. The methods are detailed below, and the results shown in Table 3.8.

Ultrafiltration

The 1000ml of virus suspension was prefiltered through a 0.22um cartridge filter to remove cellular debris which would block the system. Two Amicon hollow fibre cartridges (1000 molecular weight cut-off) were connected to a peristaltic pump and the virus suspension pumped through. The waste and the concentrate were sampled for titration. The process took approximately 3-4hrs.

Ammonium sulphate precipitation (Felmingham and Brown, 1977)

1500ml of sterile saturated $(NH_4)_2SO_4$ in TNE were added to 1000ml of virus suspension. The contents were mixed thoroughly and immediately decanted into centrifuge bottles and spun at 9K 4^oC for 30min. The supernatant was sampled, and discarded and the pellet was resuspended in ice cold TNE at 1/20 of the original volume. This concentrate was sampled then stored at -70^oC.

Polyethylene glycol (PEG) precipitation (Magar and Lecomte, 1987)

250ml of 50% (w/v) PEG 6-8000 (in TNE) were added to 1000ml of virus suspension. This gave a final concentration of 10% w/v PEG. The mixture was left at 4^oC overnight with vigorous stirring, then spun at 9K, 30min, 4^oC, to collect the precipitate. The supernatants were sampled and discarded and

the pellets resuspended in 1/20 volume of ice-cold TNE. The concentrate was sampled and stored at-70°C.

Results of virus concentration: Table 3.8

A. Ultrafiltration	$TCID_{50}$/ml	Volume (ml)	Recovery
Starting supernatant	7.2×10^5	1000	
Prefiltered supernatant	4×10^5	1000	53%
Waste from ultrafiltration	4×10^4	~ 1000	
Concentrated supernatant	4×10^5	110	6%
B. $(NH_4)_2SO_4$ precipitation			
Starting supernatant	7.2×10^5	1000	
Waste supernatant	2×10^5	2500	
Virus concentrate in TNE	2.2×10^6	50	15%
C. PEG precipitation			
Starting supernatant	7.2×10^5	1000	
Waste supernatant	2×10^5	1250	
Virus concentrate in TNE	1×10^7	50	70%

The ultrafiltration apparatus was clearly faulty.

Thus PEG precipitation gave a far superior recovery of virus to the other two methods tested. In addition it was simple to perform, and this was therefore chosen as the method of concentrating the 25l of stored virus supernatant.

Determination of optimum purification procedure

In previous reports on BVDV (Felmingham and Brown (1977), Prichett and Zee (1975), Purchio *et al* (1983)) the virus had been purified by sucrose gradient sedimentation. Some of these reports describe rate zonal sedimentation to band virus, but

this has the disadvantage that the band tends to be diffuse, due to the large range of densities of pestivirus virions (Horzinek, 1981). In the following experiment different sedimentation procedures were compared for efficiency of virus recovery.

A. Direct pelleting of virus from concentrate

20ml of PEG-concentrated virus in TNE were spun at 25K for 16hr at 4°C in an sw28 rotor (Beckman). The pellet was resuspended in TNE for titration.

Results	Titre ($TCID_{50}$/ml)	Volume (ml)	Total
PEG supernatant	1×10^7	20	2×10^8
Pellet	8×10^7	2	1.6×10^8
Recovery =	80%		

B. Centrifugation through discontinuous sucrose gradients before pelleting

Sucrose gradients were 3ml each of 35% and 20% sucrose (w/w) in TNE in an SW40 tube. These were overlaid with 5ml of virus concentrate in TNE and spun at 20K 2hrs. This should pellet cellular material while leaving the virus at the interface between the two sucrose concentrations. The fraction was then re-spun to directly pellet the virus as described in procedure A.

Results	Titre ($TCID_{50}$/ml)	Volume (ml)	Total
Starting virus	1×10^7	5	5×10^7
Sucrose interface	6×10^6	1	6×10^6
Second pellet	5×10^6	1	5×10^6
Supernatant	2.1×10^5	12	2.5×10^6
Recovery =	10%		

C. Rate zonal banding of virus

Pellets of virus obtained in procedure A were resuspended in TNE and layered onto a 10-40% continuous sucrose gradient in TNE. The gradients were spun at 25K 4°C for 20hrs in an SW40 rotor and fractionated. Fractions were analysed specrophotometrically.

Results	Titre ($TCID_{50}$/ml)	Volume (ml)	Total
Starting virus	8×10^7	500	4×10^7
Virus banded (2 fractions)	1×10^7	1	1×10^7
Recovery =	25%		

Thus a single pelleting out of suspension gave by far the best recovery. It was felt that the recoveries from the gradient purifications were too poor to justify their use and it was decided simply to pellet the virus directly out of the PEG concentrate, since the most crucial factor was the amount of virus obtainable, rather than its purity.

3.4.2 Large scale concentration and purification of BDV

Twenty-five litres of tissue culture supernatant containing BDV had been stored at -70°C following virus harvest as described in Chapter 2, Section 2.3. Two litre batches were defrosted at room temperature, pooled and sampled for titration. These were precipitated overnight by PEG, and concentrated as described previously. The PEG concentrate in TNE was then spun at 25K 20hrs at 4°C to pellet the virus out of suspension. Virus pellets were immediately frozen at -70°C until all of the 25l had been processed. The pellets were now ready for the isolation of viral RNA.

Titres are shown below.

Large scale purification of BDNCP virus

Fraction	Mean pooled titre	Volume	Total virus	Recovery
Culture supernatant	5×10^5	25l	1×10^{10}	
PEG concentrate	5×10^6	1250ml	6×10^9	60%

At an estimated 80% recovery of virus during the next direct pelleting stage, there should be approximately 5×10^9 total

virus in the frozen pellets. Working on the results obtained by Renard *et al* (1985), where 6 x 10^{10} $TCID_{50}$ of BVDV yielded 1ug of virus RNA, this amount of BDV should yield a maximum of 80ng of RNA.

3.5 SUMMARY

The growth of BDV was studied in the presence of AcD to assess this drug's usefulness in enhancing BDV growth on a large scale. Although AcD did enhance BDV replication during the initial stages of an infection there was no evidence that it led to any increase in final virus titre. It was not therefore used in large scale BDV growth. However, it did prove useful in experiments studying the replication of viral RNA *in vivo*. These experiments failed to detect labelled precursor in mature virion RNA, but were able to show early synthesis of an AcD-resistant fraction of RNA, presumably viral.

Large scale production of BDV was difficult owing to the poor replication of virus in tissue culture and the consequent large volumes of culture fluid required. Twenty five litres of culture supernatant yielded approximately 5 x 10^{9} $TCID_{50}$ of virus after PEG concentration and virus pelleting. The virus was stored at -70^{o}C until use.

CHAPTER 4

ISOLATION AND ANALYSIS OF BDV GENOMIC RNA AND ITS USE IN cDNA CLONING

4.1 INTRODUCTION

The genome of pestiviruses has been shown to consist of a single molecule of positive stranded RNA. Until relatively recently the size of this genomic RNA remained unclear. Renard *et al* (1985) published a report sizing the BVDV (Osloss strain) RNA at 12.5kb. The NADL strain of BVDV was shown to have an RNA of similar size (Collett *et al*, 1988c), as were several other BVDV strains (Collett *et al* 1988a). HCV genomic RNA has also been reported to have a length of around 12kb (Moorman *et al*, 1990). There have been no reports in the literature concerning the size of the genomic RNA of BDV.

It has also been suggested that pestivirus genomic RNAs have extensive secondary structure. This idea arose from their sedimentation data, for which values between 24s and 40s have been reported. Experiments by Collett *et al* (1988c) revealed that the BVDV RNA has properties which would be consistent with areas of secondary structure, including the binding of the RNA to CF-11 cellulose, resistance to mild RNAase treatment, and solubility in 2M LiCl. This last property was exploited by Collett's group to purify BVDV RNA from infected cells. This chapter begins with a section on the use of these techniques to prepare BDV RNA from infected cells. The next section describes attempts to prepare ^{32}P labelled RNA from BDV virions labelled *in vivo*, and the last section describes the purification of BDV RNA from virions on a large scale, prior to cDNA cloning.

4.2 ISOLATION AND ANALYSIS OF BDV RNA FROM INFECTED CELLS

When Collett *et al* (1988c) published a method describing the purification of BVDV RNA from infected cells, it was decided to utilize the cell monolayers from the large scale virus production (Chapter 3, Section 4) as another potential source of BDV genomic RNA. The problems encountered with this approach

were again related to the poor replication of virus in tissue culture, additionally the very large size of the RNA molecule renders it extremely susceptible to degradation by RNAses. The RNA of pestiviruses is also difficult to purify from cell-associated fractions, presumably because its secondary structure results in a diffuse sedimentation pattern.

4.2.1 Experimental

RNA was prepared from 10 roller cultures of FLM cells infected with BDNCP (G1480). This was done in two batches. The cells were removed from the glass by trypsinisation in trypsin/versene 1/5 at 37°C. Cells were washed twice in ice cold TNE and pelleted at 4°C. The pellets were resuspended in TNE containing 0.1% SDS, and the suspension was extracted with phenol as described in 2.4.3. Following two ethanol precipitations this total cytoplasmic RNA fraction was dissolved in 2M LiCl /1% SDS in 10mM EDTA. The RNA was left overnight at -20°C to precipitate the bulk of the cellular fraction. The solution was centrifuged at 12K for 30min in a Beckman Ja-20 rotor in Corex tubes, at 4°C. The supernatant was decanted into a fresh tube and precipitated with 3 volumes of ethanol. The invisible pellet was resuspended in 20ul depc-treated water, ready for electrophoresis on a denaturing formaldehyde gel. A control RNA fraction was prepared in the same way from uninfected FLM cells. The RNAs were run on a denaturing formaldehyde gel (Chapter 2, Section 5.2) alongside size markers to determine the molecular weight. The results are shown in Figure 4.1.

Although ethidium bromide staining of RNA in formaldehyde gels is poor, a faint high molecular weight band was visible from the virus infected cells (track 4). This was assumed to be the BDV genomic RNA on the basis of its size and the absence of a similar band in the negative control (track 3). Unfortunately, limited amounts of material prohibited a DNAase digest on the high molecular weight fraction to eliminate the possibility that it was contaminating DNA.

Figure 4.1 Denaturing agarose gel electrophoresis of the LiCl soluble fraction of RNA from BDNCP infected cells.

The gel was 0.75% agarose.

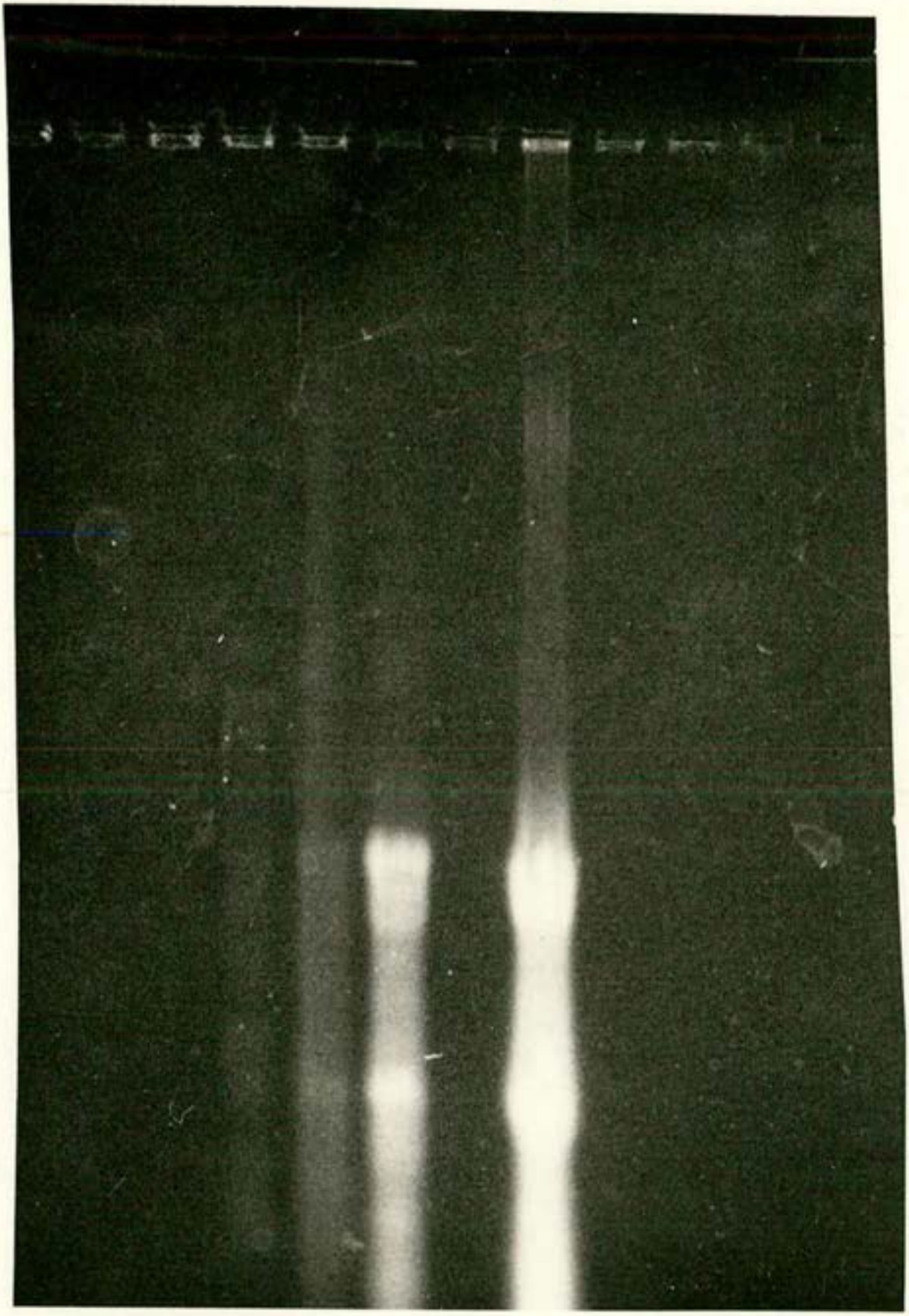
Tracks: 1. 0.5ug RNA ladder (BRL)

2. 0.5ug 28s/18s rRNA markers (mouse liver)

3. LiCl supernatant RNA fraction from uninfected FLM cells

4. LiCl supernatant fraction from BDNCP infected FLM cells

1 2 3 4



Estimation of the molecular weight of BDV genomic RNA

A standard curve was plotted of log mol wt vs mobility, using the RNA ladder molecular weight standards (BRL). This plot is shown in Figure 4.2. The migration point of the high molecular weight RNA was 40mm as indicated by the arrow. From this it was deduced that this RNA was 12.5-13kb in size. This is in agreement with sizes of other pestivirus RNAs (Moorman *et al*, 1990; Collett *et al*, 1988b; Renard *et al*, 1985). The yield of this viral genomic RNA (as deduced by the intensity of UV fluorescence) was approximately 50ng from ten infected monolayers (1×10^8 cells), thus it was not judged to be a feasible route to preparative amounts of viral RNA, as we would have needed 10 times this amount and time was limiting.

4.3 PRODUCTION OF LABELLED BDV *IN VIVO* USING ^{32}P

Despite unsuccessful attempts to produce progeny virion RNA labelled with the precursor ^{3}H uridine it was decided to attempt a further *in vivo* virus labelling experiment, this time using ^{32}P. The rationale was that firstly, by using ^{32}P orthophosphate in the culture medium, a much higher specific activity could be achieved than with ^{3}H, which may allow incorporation of label into virus particles. Secondly, the sensitivity of detection of ^{32}P labelled material is vastly superior to that of ^{3}H labelled material, and given the low levels of virus genomic RNA available this increased sensitivity might allow detection of labelled virus RNA.

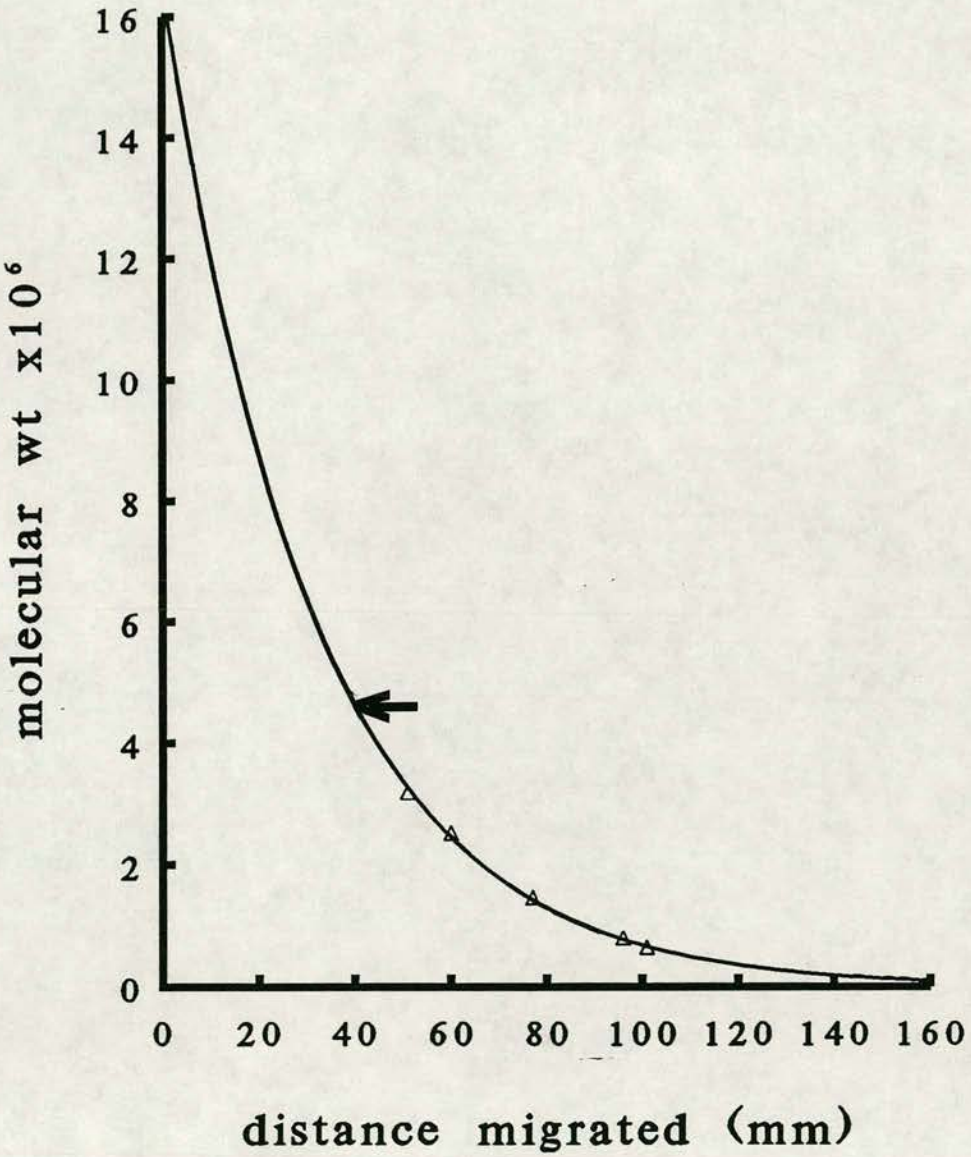
4.3.1 Experimental

The same criteria applied as for the ^{3}H labelling experiment (Chapter 3, Section 3) in that the label had to be available inside the cell, ie the intracellular pool was at equilibrium, and that host cell incorporation of label into RNA was minimised by AcD. However, the precursor used in this experiment was

Figure 4.2 Standard curve to determine the molecular weight of the large RNA shown in Figure 4.1. Plot of log molecular weight of RNA standards (BRL) vs mobility in the gel. The position of the high molecular weight RNA is indicated by the arrow.

This corresponds to a molecular weight of approximately 4.5×10^6 kd. Assuming one nucleotide to average m wt 330, this is equivalent to size of 13.6 kb.

Standard curve:RNA mobility. Mobility of RNA standards in denaturing gels.



inorganic ^{32}P orthophosphate, which will produce highly labelled cells and virus, since it will be incorporated into every phosphate-containing compound, notably DNA during cellular division, although cell growth is minimal in confluent cultures. Nevertheless, all material will be highly labelled and difficulties may arise in purifying the labelled virus RNA away from the enormous background of cellular labelled material.

Four confluent cultures of FLM cells grown in $75cm^2$ flasks were used in the experiment. Two cultures were infected with BD NCP as described previously, and two were left as uninfected control cultures.

One infected and one uninfected culture were labelled with ^{32}P as follows: cultures were incubated in 25ml phosphate-free maintenance medium to which carrier free ^{32}P orthophosphate had been added, at 0.5mCi/flask. Maintenance medium was phosphate free E199 medium (GIBCO custom-made medium) with 2% dialysed FBS and 5ug/ml AcD. Cultures were incubated for 40mins in the presence of label. The highly radioactive supernatant medium was processed as for virus harvesting (Chapter 2, Section 2.3) and discarded into cold carrier phosphate solution. The unlabelled culture was harvested in parallel and a sample was taken for titration, to indicate the amount of virus present. "Virus pellets" from infected and uninfected cultures were processed to yield RNA and the proportion of labelled RNA was determined. Pellets were resuspended in TNE containing SDS (0.1%) and proteinase K at 200ug/ml and incubated $37^{o}C$ 30min. This digest was extracted twice with phenol and once with chloroform, and ethanol precipitated. The precipitated material was resuspended in TE/SDS (0.1%) and the proportion of TCA insoluble counts was measured.

A sample of this labelled RNA fraction was run on a denaturing formaldehyde gel (Chapter 2, Section 5.2) and another sample on a 10-30% sucrose gradient which was fractionated and the fractions precipitated and counted using Cerencov counting.

4.3.2 Results

(a) Titration of unlabelled, infected control cultures

This was done by an immunoperoxidase titration as described in Chapter 2, Section 2.3. Assuming that the label has no effect on virus replication, then these figures should reflect the titre of the labelled virus culture. The results are summarised in Table 4.1.

(b) Incorporation of ^{32}P into TCA insoluble material

Virus pellets from infected and uninfected cultures were resuspended in TNE, 0.1% SDS with proteinase K at 200ug/ml. After incubation at 37°C, 30 min, this digest was extracted twice with phenol, once with chloroform and precipitated with ethanol. The precipitate was resuspended in TNE, 0.1% SDS and the proportion of counts insoluble in 5% TCA was measured. The results are shown in Table 4.2.

(c) Denaturing gel electrophoresis of the labelled RNA

Infected and uninfected labelled RNA fractions were run on a formaldehyde gel. The gel was dried and an autoradiograph produced as described in Chapter 2, Section 6.6. The results are shown in Figure 4.3.

(d) Sucrose gradient profiles of 32P labelled RNA from BDNCP infected and uninfected cultures

Sucrose gradients were prepared, run and fractionated as described in Chapter 2, Section 4.6). 2×10^5cpm of ^{32}P labelled RNA from an infected and uninfected culture were run on parallel 10-30% continuous gradients. The run conditions were 20hrs at 20,000rpm at 20°C in an sw40 rotor (Beckman). 0.5ml fractions were collected, ethanol precipitated and resuspended in 50ul TE. 25ul were spotted onto a filter and counted in PPO scintillator fluid for 1 minute. The gradient profiles are shown in Figure 4.4.

Table 4.1

Titration of unlabelled control cultures

	Titre	Volume	Total
Culture supernatant after clarifying	1×10^5	25ml	2.5×10^6
Culture supernatant after pelleting	1×10^4	25ml	2.5×10^5
Virus pellet	1×10^6	2.0ml	2.0×10^6

Therefore the virus pellet from a 25ml culture should contain 1×10^6 $TCID_{50}$.

Table 4.2

Incorporation of label into the TCA insoluble fraction

	Infected culture	Uninfected culture
Total cpm	599 5052	348 579
TCA insoluble cpm	870 351	30 711
% incorporation	14%	9%

Thus 1×10^6 $TCID_{50}$ of virus from above yielded RNA labelled with 8×10^5 cpm.

Figure 4.3 Autoradiograph of ^{32}P labelled RNA prepared from BDNCP infected and uninfected cultures by labelling *in vitro*. (Cellular 28s and 18s fractions are indicated by the arrows)

Track: 1. 4×10^4 cpm from the uninfected culture

2. 5×10^4 cpm from the BDNCP culture

3. 5×10^5 cpm from the uninfected culture

4. 5×10^5 cpm from the BDNCP culture

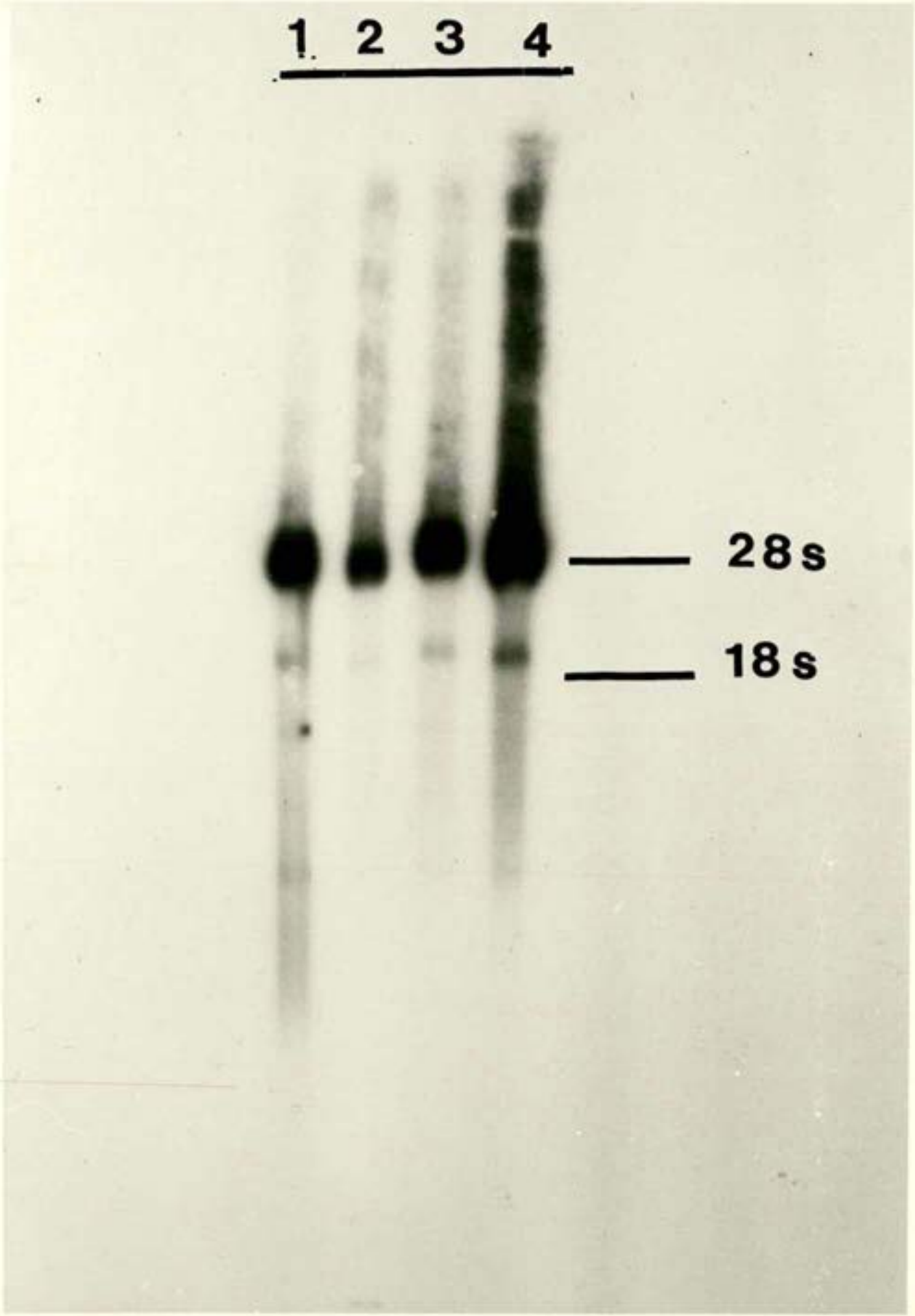
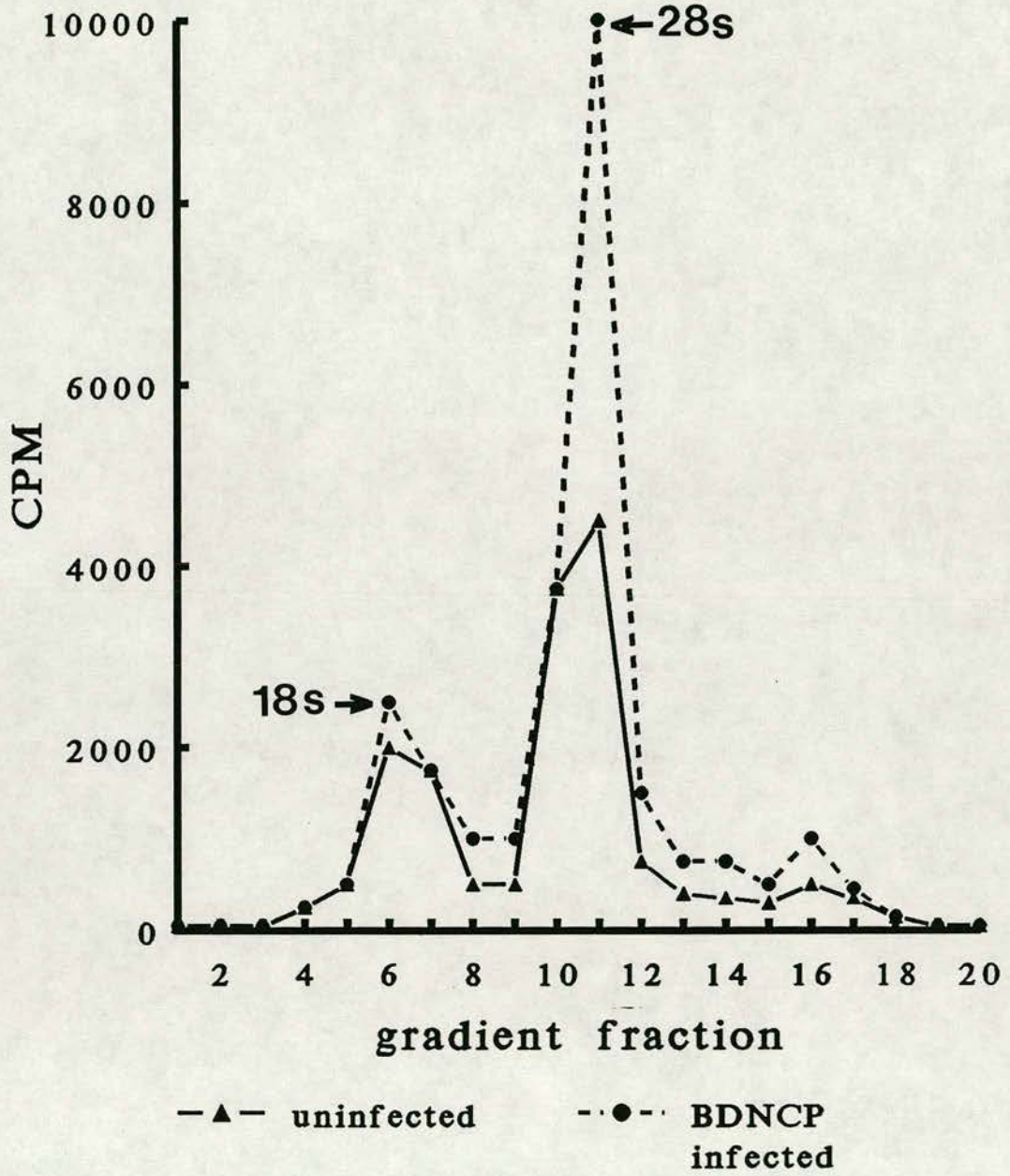

1 2 3 4
28s
18s

Figure 4.4 Gradient profile of ^{32}P labelled RNA from BDNCP infected cells labelled *in vivo*.

Gradient profile of RNA from ^{32}P labelled BDNCP infected cells



4.3.3 Conclusions

BDNCP was grown to a titre of 1 x 10^5 $TCID_{50}$/ml in phosphate free medium containing ^{32}P orthophosphate for 40hrs. Harvesting the virus from these cultures yielded 1 x 10^6 $TCID_{50}$. From this virus pellet RNA was prepared and the incorporation of label into the TCA insoluble fraction was measured. The RNAs of both the infected and uninfected cultures were highly labelled, as expected since orthophosphate is a non-specific precursor. Allowing for this background, 1 x 10^6 $TCID_{50}$ of virus yielded 8 x 10^5cpm of labelled RNA. When RNA was analysed by denaturing formaldehyde gel electrophoresis a large amount of contaminating cellular material was obvious both in infected and uninfected cultures. There appears to be slightly higher molecular weight material visible in the infected RNA sample but this did not appear as a discrete band. In this infected sample, labelled material was present up to 10kb. The same RNA samples were analysed by rate zonal sedimentation through sucrose density gradients and a small peak was present solely in the infected fraction around the 35s region of the gradient.

On the basis of these experiments it is probable that the BDNCP genomic RNA was demonstrated. However, lack of material meant that DNAse digestion controls were not performed to eliminate the possiblity of contaminating DNA. It is also possible that the high molecular weight fractions observed in gradients are aggregates of cellular rRNA since a discrete band was not obtained.

4.4 LARGE SCALE PRODUCTION OF BDV GENOMIC RNA FOR USE IN cDNA REACTIONS

RNA was to be prepared from the 5 x 10^9 $TCID_{50}$ of BDNCP stored as frozen virus pellets. This RNA would then be purified by rate zonal sedimentation through sucrose density gradients. The problem was the lack of detection system available for BDV RNA. The amount of RNA obtainable (80ng) would be far too small to permit a spectrophotometric gradient profile. The only

option appeared to be to use a suitable probe to hybridise across the gradient, however, no probes showed unequivocally specific hybridisation to BDV RNA. It was therefore decided to use a parallel RNA purification from BVDV-NADL virions to which there were numerous specific DNA probes available. The NADL RNA was then fractionated on a parallel gradient with the BDNCP preparation, and the labelled probe was hybridised across the NADL gradient to detect the virus RNA-containing fractions. The equivalent fractions from the BDNCP RNA gradient were then pooled and assumed to contain whatever BDNCP RNA was present. This relied on two assumptions:

(a) that the BDNCP RNA is approximately the same size as the BVDV-NADL RNA, and

(b) that the BDNCP RNA had not been substantially degraded.

4.4.1 Experimental

Approximately 6×10^9 $TCID_{50}$ of BVDV-NADL were spun from PEG concentrated tissue culture supernatant at 20k for 16hrs. The virus pellets were stored at -70°C until required. 6×10^9 $TCID_{50}$ of BDNCP which had been grown on a large scale (Chapter 3) were used as the source of BDNCP RNA. RNA was prepared from both sets of virus pellets by thawing the pellets directly into Guanidinium isothiocyanate RNA isolation buffer, and purifying through a 5.7M CsCl cushion as described in Chapter 2, Section 4.5). The RNA pellets were precipitated with ethanol and redissolved in sucrose gradient loading buffer (1 x 7E pH 7.5/0.1% SDS). Low salt concentrations were used throughout the gradients to minimise RNA secondary structure. The BVDV-NADL RNA was loaded onto one 10-30% continuous sucrose gradient (in TE/SDS) and the BDV sample onto a parallel gradient. Gradients were spun at 20,000rpm for 20hrs in an SW40 rotor (Beckman). Both gradients were fractionated into 0.5ml fractions. The BDV fractions were stored at -20°C. The BVDV-NADL fractions were ethanol precipitated and redissolved in 10ul depc-treated H_2O ready for denaturation and analysis by hybridisation.

Hybridisation of NADL-specific probes across the RNA gradient

The samples were denatured in formaldehyde at 65°C for 15min and loaded onto a nitrocellulose membrane using a slot-blot manifold (Chapter 2, Section 6.1). Blots were dried and fixed at 80°C for 2hrs under vacuum. They were pre-hybridised for 2hrs as described in Chapter 2 and then hybridised using 50% formamide at 42°C overnight. The probe was an antisense RNA probe produced by *in vivo* transcription of a small 192bp segment of the BVDV gene for the p80 protein. (For further discussion of this probe see Chapter 5.) The blot was washed at high stringency using 0.1 x SSC 0.1% SDS at 60°C for 2 x 30 min, and exposed to x-ray film with intensifying screens at -70°C for 2hrs. The results are shown in Figure 4.5

The high molecular weight fractions showing a good signal were presumed to contain the NADL RNA. The equivalent fractions (20-24) of the BDV RNA gradient were pooled, precipitated and stored under ethanol at -70°C until required for cDNA synthesis.

4.5 cDNA SYNTHESIS

4.5.1 Introduction

The difficulties involved in producing cDNA clones of pestivirus sequences are considerable. To a great extent the success of any cDNA cloning experiment depends on the amount and the quality of the input RNA for the template. Pestivirus growth in tissue culture is poor, leading to low virus yields and furthermore virions are difficult to purify from cellular material (Laude, 1979). This means that the source of virion RNA is extremely limited for all pestiviruses.

The second problem associated with producing high quality pestivirus RNA for cloning concerns the RNA itself. The pestiviral genomic RNA is 12.5kb in length, and its size makes it extremely vulnerable to attack by ribonucleases. It also seems to have an extensive secondary structure which may prevent certain areas of the genome being available for reverse

Figure 4.5 Autoradiograph showing slot-blot hybridisation of probe pBV4/p80 to 0.5ml fractions of a sucrose gradient containing RNA from BVDV-NADL infected cells.

Sample 23 was assumed to be a false negative due to loss of sample.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transcription, in particular the termini of the RNA. Furthermore, pestivirus RNA is not polyadenylated and therefore cannot be purified using oligo-dT chromatography: the starting preparations of RNA will therefore be fairly crude. Thus the scarcity, fragility and inherent structure of these viral RNAs have all conspired to prevent any success in cDNA cloning until relatively recently (Renard *et* 1985). There are now several reports of BVDV and HCV cloning and none of BDV.

4.5.2 Strategies for cDNA production and cloning

The initial decision was whether to purify viral RNA from total infected cellular RNA or from purified or semi-purified virions. Both approaches have been used with success. Moorman *et al* (1990) and Collett *et al* (1988b) have both taken the intracellular RNA approach while Rumenapf *et al* (1989), Brock *et al* (1988) and Renard *et al* (1985) have all used RNA purified from extracellular virions which were pelleted from tissue culture fluid.

Having isolated the template RNA the next decision was how to prime synthesis of the first strand of cDNA. Since the pestivirus RNA has no poly-A tail there are 3 options. A poly A tail could be added synthetically. This approach was used by Brock *et al* (1988) and Renard *et al* (1985). Their reason for using this approach was presumably because they planned to clone by homopolymer tailing, which requires the poly dT/dA stretch produced by oligo dT priming. This seemed an unnecessarily complicated approach involving many manipulations of a scarce and fragile RNA. The other means of priming was using random hexanucleotides or site-specific oligonucleotides. The latter had the advantage of allowing selective cloning of areas of interest, but needed exact sequence data in order to design primers. The simplest and most suitable method appeared to be random priming of first strand cDNA using hexanucleotide primers. This method sacrificed selectivity in an already heterogenous RNA population, since cellular RNA will co-purify with the pestivirus RNAs. However, the method had been used

successfully by several groups (Rumenapf *et al*, 1989; Renard *et al*, 1985, Collett *et al*, 1988c, Moorman *et al*, 1990) and it was decided on as the method of choice. First strand cDNA would be converted to a double-stranded molecule by the standard method of Gubler and Hoffman (1983).

The next decisions concerned the cloning strategy. The options were: linker ligation or adaptor ligation involving attachment of linkers/adaptors to double stranded blunt-ended cDNA, or simple blunt ended ligation into a blunt-ended vector. The homopolymer tailing method of cloning was not available because it requires a poly dT/dA stretch on the cDNA produced by oligo dT priming. It was decided to simply blunt end the double stranded cDNA and clone this directly into a blunt-ended restriction site in the vector. This would avoid risks of loss of the small amounts of cDNA produced on 80ng of template. Fewer manipulations should result in conserving the small amount of cDNA produced.

The vector chosen for cloning was Bluescribe. This is a 2kb plasmid vector which allows easy colour selection of recombinants.

Bluescribe would be digested with *Sma*I to produce a blunt-ended vector and ligated to the blunt-ended cDNA. Clones would be selected by colour and grown for screening. Since we had no BDV specific clones, the initial screening stage would involve a plus/minus screen using cDNA produced from total RNA from virus infected and uninfected cells. A flow summary of the cloning approach is shown in Figure 4.6.

4.5.3 Experimental

First strand cDNA synthesis was performed using cloned MMLV-RT enzyme lacking RNAse H activity (BRL) (Gerard *et al*, 1990). The BDNCP RNA was mixed with 0.8ug of random hexanucleotide primers (Stock = 0.8mg/ml) (Pharmacia). The mixture was adjusted to 14ul with water, heated to 70^{o}C for 10

min then chilled on ice. The first strand reaction components were added as follows; 5 x reaction buffer (supplied with enzyme) to 1x, 0.1M dithiothreitol (DTT) to 0.01M, dNTPs to 0.5mM each, 1ul of RNA guard (Pharmacia) and 200 units of MMLV RT. The final volume was 25ul. 5ul were removed and incubated in the presence of ^{32}P dCTP (2uCi). The remaining 20ul were not labelled. Incubation of both reactions was at 42°C for 1hr. The 20ul reaction was then diluted into a second strand reaction mixture (~ 150ul) as follows:

91.8ul H_2O
32ul 5 x second strand buffer
3ul 10mM dNTP mixture
6ul 0.1M DTT
5uCi ^{32}P dCTP
4ul (40u) *E. coli* DNA pol 1 (Pharmacia)
1.5 units RNAse H (BRL)

The reaction was incubated for 2hrs at 16°C. 10 units of T4 polymerase were added and incubated a further 30min at 37°C.

Figure 4.6 Experimental strategy for production of BDNCP cDNA clones.

Starting material = 5 x 10^9 $TCID_{50}$ of frozen BDNCP virus pellets

Prepare virus RNA from pellets and purify through CsCl

Further purify virus RNA on sucrose gradients alongside BVDV RNA in a parallel gradient

Fractionate BVDV gradient and identify virus RNA fractions by probe hybridisation. Pool and precipitate the corresponding fractions from the BDV gradient

Commit half this BDV RNA to a 1st strand cDNA synthesis reaction using murine moloney leukemia virus reverse transcriptase (MMLV-RT) and random hexanucleotide primers

Second strand cDNA synthesis using DNA pol I and RNAse H

Blunt end cDNA using T4 polymerase

Direct blunt-end ligation of the cDNA into *Sma*I cut Bluescribe vector

Select recombinants by colour and grow for screening

Produce cDNA probes using ^{32}P and reverse transcriptase on total cellular RNA from infected and uninfected cells. Probe colonies with these probes in a +/- screen.

Estimation of yield of cDNA

The small (5ul) reaction was labelled with 2uCi. 1ul of this was diluted 1/10 into carrier DNA (10ng/ml salmon sperm DNA) and 1ul of this was spotted onto a Whatman glass fibre disk. Counts were measured before and after TCA washes (5 x 10secs in 10% TCA). The incorporation of label was 4%. Theoretical yield of cDNA is 13.2ug (40n moles of dNTP in 20ul reaction). Actual yield = 13.2 x 0.04 = 0.5ug cDNA.

Second strand yield

Incorporation of label was 5%.

Yield of second strand = 0.05 x (13.2 - 0.5ug 1st strand)

= 0.63ug second strand cDNA produced

cDNA cloning

It was decided to attempt to clone "blind" the products of the second strand synthesis reaction, because the amounts of cDNA, if present, were far too small to allow the reaction to be analysed on gels.

Preparation of Bluescribe vector for cloning

5ug of Bluescribe vector was digested with *Sma*I restriction enzyme as described in Chapter 2. The digested vector was extracted twice with phenol chloroform and precipitated twice with 2.5 volumes of ethanol. The pellet was resuspended in TE pH8.0 at 0.1mg/ml.

Ligation

The expected efficiency of direct blunt-ended ligation into *Sma*I cut Bluescribe was tested using control blunt-ended DNA fragments produced by Hae III digestion). The control blunt-ended DNA was adjusted to 0.1ug/ml in TE.

Ligations were set up as follows:

0.1ug vector (unphosphatased)
0.1ug blunt-ended fragments
1ul 10 x T4 ligase buffer
(250mM Tris 50mM $MgCl_2$ 5mM ATP)
3.75ul 40% PEG_{8000}
1ul 5mM ATP

Adjusted volume to 10ul with H_2O and incubated at 16°C overnight.

The ligation reactions were diluted and transformed into competent *E. coli* JM109 cells. Transformation was carried out as described by Chung *et al* (1989). Transformation efficiency of closed circular plasmid DNA was between 5 x 10^7 and 1 x 10^8 colonies per microgram of DNA. Transformants were selected as described below.

Selection of transformants and recombinants

Selective medium consisted of L-agar containing 50ug/ml Ampicillin, 40ug/ml X gal and 5mM IPTG. Cells containing non-recombinant plasmid gave rise to blue colonies while recombinants gave rise to white colonies.

Ligation efficiency

Ligation/transformations were set up as decribed above. Results are shown below.

Ligation results

Plate	Received	Blue colonies	White colonies
1	No DNA	0	0
2	1pg uncut BS	80	0
3	10pg uncut BS	500	0
4	100pg uncut BS	> 2000	0
5	100pg self ligated BS	25	0
6	100pg BS + 100pg Hael fragments	18	10

Each plate contains 1/10 of the transformation mix.

The self-ligated vector reactions appears to contain 100-fold less covalently closed circular plasmid than the control, as does the *Hae*I ligation reaction. Thus for both intra- and inter-molecular ligation of blunt ends, efficiency appears to be approximately 1% , although a linear plasmid was not transformed as a control.

Ligation and transformation of putative BDNCP blunt-ended cDNA

Assuming 1% ligation efficiency as described above. Input DNA was 100ng of vector and half of the total 2nd strand synthesis reaction. The ligation reaction was set up and transformed and recombinants selected, all as described in the preceding section.

Results - Table 4.3

Transformation efficiency was 2.5×10^7 and the ligation efficiency was again around 1%. Unfortunately no recombinant colonies were obtained from the BDNCP cDNA ligation. This experiment was repeated with the last batch of the cDNA reaction, but no recombinant colonies were obtained. The reasons for this failure are not clear, the system appeared to be working as judged from the controls, and the problem seemed therefore to be inherent in the cDNA reaction. Either there was too little, if any, second-strand cDNA present (as suggested by the alkaline gel results) or what was there was unclonable perhaps due to poor efficiency of blunt-end production.

4.6 SUMMARY

Based on published results with BVDV (Renard *et al*, 1985) the theoretical yield of pestivirus RNA from pelleted virions is lug viral RNA from 1×10^{11} $TCID_{50}$ of virus. Although we had only a total of 5×10^9 $TCID_{50}$ of BDNCP virus a cDNA synthesis and cloning experiment was attempted. The theoretical yield of BDNCP RNA was 80ng. This was split into two batches and two attempts at cDNA synthesis were performed. On both occasions some high molecular weight products were detected in the first

Table 4.3

Ligation of BDNCP cDNA and Bluescribe vector

Plate		Colonies	
		Blue	White
1	No DNA	0	0
2	10pg uncut ccc BS	50	0
3	Self ligated BS 100pg	30	0
4	BDNCP cDNA/100ng linear BS	400	0

strand reactions but nothing was visible on an autoradiograph of the second-strand reaction. Despite this, the products of each second-strand reaction were ligated to linearised unphosphatased bluescribe vector in a simple blunt-ended ligation without using linkers or adaptors. The ligation and transformation reactions were successful but yielded no BDNCP recombinants. This was judged to be due either to lack of material or of failure to produce a clonable blunt-ended second-strand product. The most likely explanation is that there was never enough starting material at every stage, and this resulted in a failure to produce recombinant BDNCP clones.

CHAPTER 5

SPECIFICITY OF CLONES IN HYBRIDISATION STUDIES

5.1 INTRODUCTION

A range of cloned cDNAs representing BVDV sequences were available. Details on these clones are given in the following section, and where the clones map on the BVDV genome is shown in Figure 5.1.

This chapter describes experiments to test six different BVDV cDNA clones for their ability to hybridise to the RNA of various BD and BVD virus strains. The purpose was to yield information on the extent and position of homology between strains and species. Additionally, this was undertaken to search for a BVDV clone which hybridises with BDV RNA, thus providing a potential probe for use in screening a BDV cDNA library, and which could also be of use to monitor BDV RNA purification.

Since there was almost no molecular information available concerning BDV, there was little idea of the extent of homology between BVDV and BDV. At the time of these experiments there was no sequence information available for HCV either, although there has since been publication of HCV sequences (Moorman *et al*, 1990, Meyers *et al*, 1989). Further discussion on sequence comparisons can be found in Chapter 7 of this thesis.

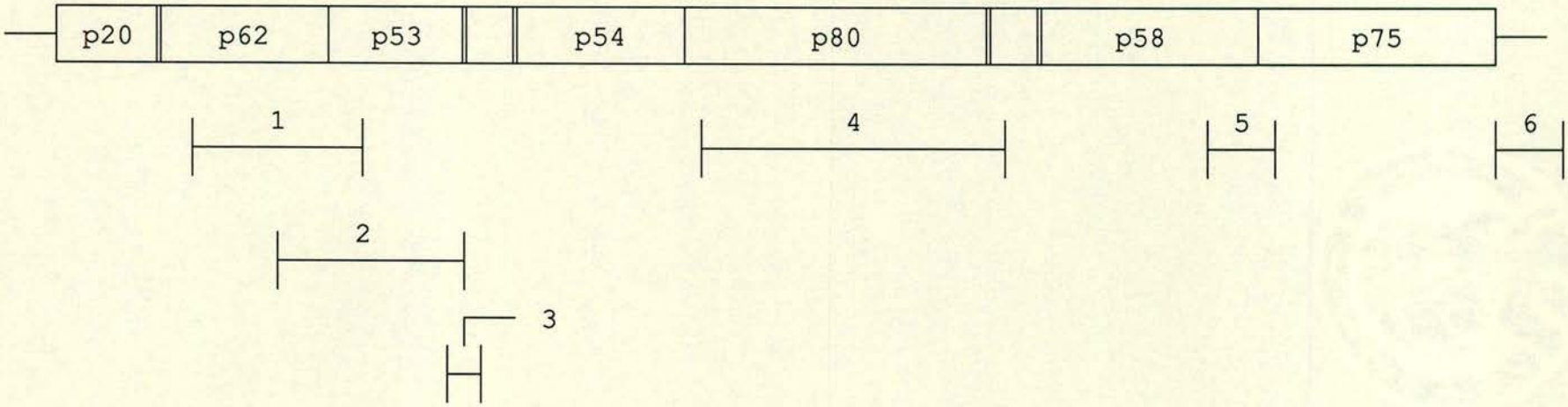
5.2 BVDV cDNA CLONES

Clones 35, 50 and 75

These were three cDNA clones produced at the Moredun Institute, Edinburgh in an earlier attempt to clone BVDV sequences (A. Herring, unpublished). Using the BVDV-NADL strain as a source of RNA, a cDNA library was constructed in pUC8, the cDNA was cloned into the *Sma*-1 site of the plasmid. The library was screened with total cDNA probes constructed from virus-infected and uninfected cells in a plus/minus screen. Several clones were then screened by removing their inserts,

Figure 5.1 Position of BVD clones on the NADL genome. Not to scale.

BVDV GENOMIC RNA



KEY

1	pN24	(1319 - 2479)	4	pBV4/p80	(5644 - 7949)
2	p503	(1800 - 3200)	5	35	(9874 - 10053)
3	50	(3008 - 3167)	6	75	(11634 - 11831)

labelling and hybridising to infected and uninfected total-cell RNA preparations. The three resulting positive clones were designated 35, 50 and 75. Each of these clones contains a short insert: clone 35 is 180bp long, clone 50 is 160bp long and clone 75 is 198bp long.

These clones have been sequenced (M. Sweeney, Warwick University, personal communcation) and mapped on the BVDV genome. Clone 50 is derived from the sequence for a structural glycoprotein (p62/p53), whilst both clones 35 and 75 map to the last segment of the non-structural coding sequences (p58/p75). For details see Figure 5.1. Thus it might be expected that clone 50 would show a more restricted hybridisation pattern than the other two.

Clones pN24 and P503

These clones were kindly supplied by M. Sweeney at the University of Warwick. They were also derived from a cDNA library constructed from the NADL strain of BVDV. The inserts are cloned into the *EcoR1* site of the plasmid "Bluescript". 503 was isolated using clone 50 described above, and is also positioned in the structural glycoprotein region of the genome. The insert is 1.4Kb long. pN24 is also derived from this region and overlaps p503. Its insert is 1.2kb long.

Clone pBV4/p80

This clone was kindly supplied by Dr. M.S. Collett, Molecular Genetics Inc., Minnesota, USA.

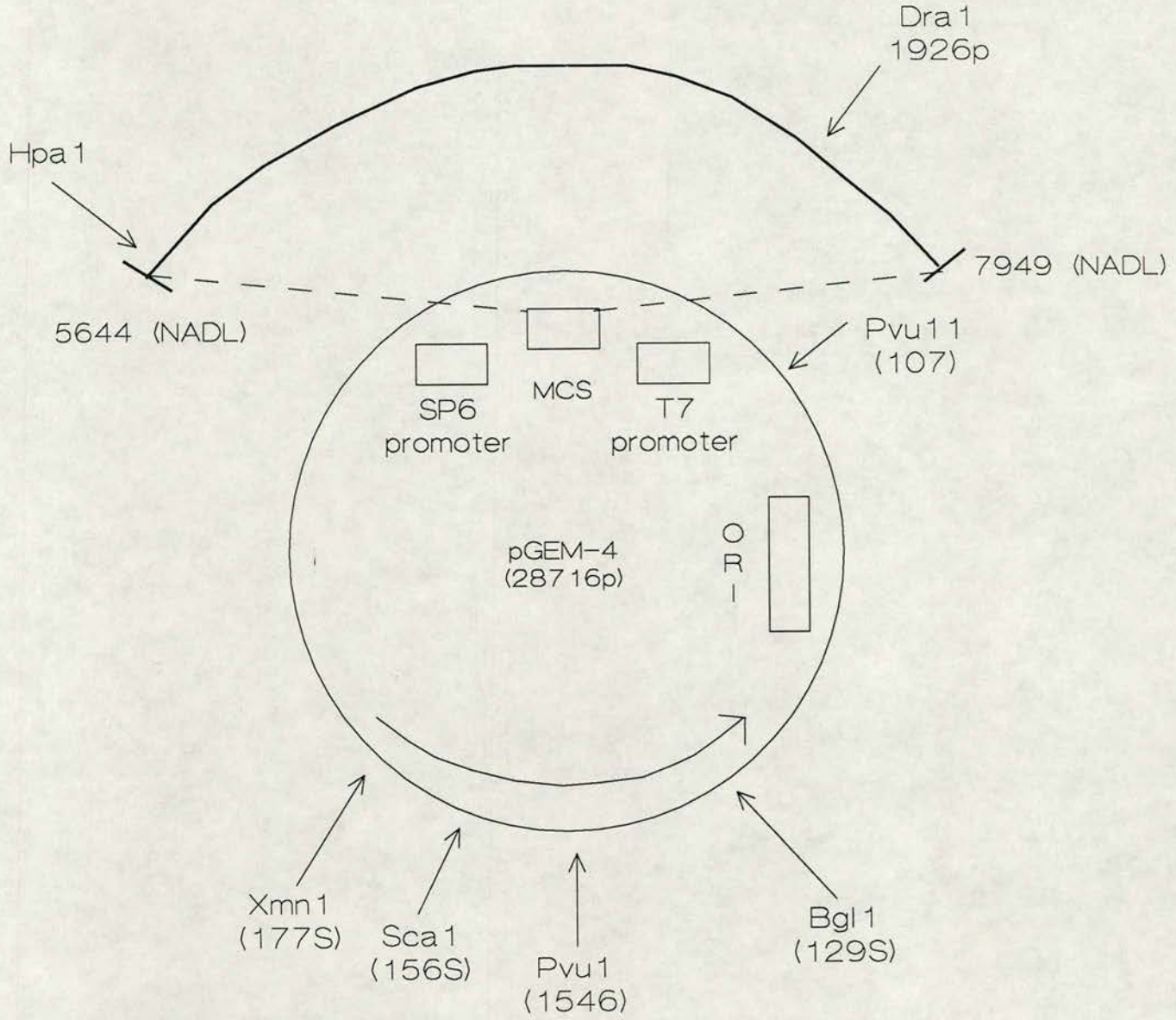
It too is derived from BVDV-NADL sequences. It consists of a 2.3kb insert cloned into the *Sma*I site of the "Gemini" transcription vector pGEM-4 (Promega Biotech). The insert spans the entire p80 region of the BVDV genome. Clone pBV4/p80 is illustrated in Figure 5.2 Antisense RNA transcribed from the T7 promoter was used as a probe.

Table 5.1 presents a summary of the BVDV clones used.

Table 5.1 Summary of BVDV clones

Clone	Vector	Insert size	Position on BVDV genome	Protein represented	Source
35	pUC8	180bp	9874-10053	p58	Dr. A.J. Herring, Moredun
50	pUC8	160bp	3008-3167	p53	"
75	pUC8	198bp	11634-11831	p75	"
p503	Bluescribe	1.4kb	1800-3200	p62	Dr. M. Sweeney, University of Warwick
pN24	Bluescribe	1.2kb	1319-2479	p62	"
pBV4/p80	pGEM4	2.3kb	5644-7949	p80	Dr. M.S. Collett, Molecular Genetics Inc., Minnesota

Figure 5.2 The clone pBV4/p80, showing unique restriction enzyme sites and the two sites used to linearise the clone for RNA probe production.


Dra 1
1926p
Hpa 1
7949 (NADL)
5644 (NADL)
Pvu 1 1
(107)
MCS
SP6
promoter
T7
promoter
pGEM-4
(2871 6p)
O
R
I
Xmn 1
(177S)
Sca 1
(156S)
Pvu 1
(1546)
Bgl 1
(129S)

5.3 EXPERIMENTAL

The experimental strategy was to grow a selection of BVDV and BDV strains and isolate total cellular RNA from the infected cells. As the virus RNA fraction of this total RNA is too small to be detected, let alone quantified, extracellular virus titre is the only indication of its presence. Therefore throughout these experiments the amount of input viral RNA could not be standardised. The detection procedure used was a dot-blot hybridisation assay as described in Chapter 2, Section 6.1. RNA from virus-infected cells was dotted onto a membrane and probed with various probes. A panel of strains was used to test seven different probes.

5.3.1 Growth of virus strains for RNA isolation

The virus strains are described in Table 5.2. Virus infection of tissue culture cells was as described (Chapter 2, Section 2.2). However, in these experiments the extracellular virus was not required other than for titration purposes, and it was clarified, aliquoted and stored at -70^{o}C as virus stock. The infected cell monolayers were washed in Hanks BSS then lysed directly into guanidinium isothiocyanate solution for RNA isolation as described in Chapter 2. Two 225cm^{2} flasks containing 100ml of medium were used for growth of each strain. The RNA was purified through a CsCl gradient and stored under ethanol until required. The extracellular virus titre for each strain grown is shown in Table 5.3

Table 5.2 Virus strains

Species	Strain	Cytopathogenicity	Origin
BVDV	NADL	CP	See Chapter 2
BVDV	Osloss	CP	
BVDV	8085	CP	Acute MD case (Highlands)
BVDV	B816	CP	Acute MD case (Borders)
BVDV	C24V	CP	
BVDV	G982	NCP	17 month-old bull with sight defect (Dumfries and Galloway)
BDV	G1480	NCP	See Chapter 2
BDV	G2048	NCP	Clinically normal survivor of BD abortion outbreak (Strathclyde)
BDV	JH2876	NCP	BD lamb (England)
BDV	L991	NCP	BD lamb (Cheshire)
BDV	D861	NCP	"Hairy Shaker" lamb (Cumbria)
BDV	Weybridge	NCP	Reference strain from CVL, Weybridge
BDV	R2727	NCP	BD lamb (England)
BDV	G1305	NCP	"Hairy Shaker" lamb (Grampian)
BDV	A1870	NCP	BD lamb (Aberystwyth)
BDV	BDV-M	CP	Vantsis *et al* (1976)

Table 5.3. Virus titres from cultures used in dot blots

Virus	Strain	Titre $TCID_{50}$/ml
BVDV	NADL	2×10^7
	Osloss	4×10^6
	8085	5×10^6
	B816	2×10^6
	C24V	1×10^7
	G982	2×10^6
BDV	G1480	4×10^6
	G2048	5×10^6
	L991	4×10^5
	R2727	2×10^5
	D861	1×10^5
	Weybridge	7×10^6
	JH2876	6×10^4
	G1305	2×10^5
	A1870	2×10^5
	BDV-M (CP)	1×10^5

5.3.2 Preparation of dot blots

The RNA preparations were quantified on an ordinary agarose gel (not shown). They were adjusted to about 1ug/10ul. Approximately 0.5ug of total cellular RNA was used per dot, but in some cases where virus titre was known to be low, this amount was increased to 1-5ug/dot in an attempt to standardise the amount of viral RNA available for hybridisation to the probe. The negative control RNA was 0.5ug/dot of uninfected FLM or BT cell RNA. In these experiments BVDV-NADL infected cell RNA was used as a positive control since all the clones tested were derived from this strain.

Harvesting of cultures infected with cytopathic (CP) strains took place when the CPE was only just becoming evident. To have let it proceed further would have reduced the number of infected cells containing viral RNA. Non-cytopathic (NCP) virus infected cultures were harvested after 72hrs. Lack of information about the kinetics and mechanism of replication of both biotypes made it impossible to define exactly the time at which there would have been the maximum amount of viral plus-stranded RNA in the cell.

5.3.3 Production of probes

Probes 35, 50 and 75 were prepared as follows. The inserts were removed from the pUC8 vector by digesting the plasmid with the restriction enzymes *EcoR1* and *HindIII*. The inserts were purified on a 2% agarose gel, (not shown). The insert band was excised from the gel and purified from the agarose by electroelution as described in Chapter 2. Electroelution was the preferred method of purification because the inserts were so small. The purified inserts were adjusted to lug/4ul, and were labelled using ^{32}P dCTP in a random-primed labelling reaction as described in Chapter 2, Section 6.3. Probes were used directly or stored for a maximum of 1 week.

Probes p503 and pN24 were prepared by digesting the plasmids with EcoR1. In the case of pN24 this gave a clean excision of the 1.2kb insert but in p503 an insert site for *EcoR1* means that

two fragments were produced at 800bp and 600bp. The larger one was chosen as a probe. The indicated fragments were excised from the gel and the DNA purified by "Geneclean", Chapter 2, Section 5.5. The purified inserts were then labelled with ^{32}P dCTP as described above to produce double-stranded DNA probes.

The last probe tested was an antisense RNA probe produced by transcription from the T7 promoter of pBV4/p80. Two RNA probes of different lengths were prepared as described in Chapter 2, Section 6.4. The potential length of probe was determined by the site at which the plasmid was linearised. In these experiments a 192bp RNA probe was produced on a plasmid template linearised by the enzyme *Dra*1, and a 2.3kb (full length) RNA probe was produced on a template plasmid linearised by *Hpa*I. Both probes were labelled using ^{32}P UTP.

5.4 RESULTS

5.4.1 Hybridisation pattern of clones 35, 50 and 75

In this experiment RNA from cells infected with a panel of BVD and BD strains was dotted onto replicate nitrocellulose membranes and probed with ^{32}P-labelled double-stranded DNA probes from clones 35, 50 and 75. Duplicate hybridisations were carried out at 42°C in 25% and 50% formamide for 16-24hrs, and the probes were at a concentration of approximately 6×10^6 cpm in 5ml of hybridisation buffer. For the actual hybridisation protocols see Chapter 2, Section 6.5. One set of filters from each hybridisation was given a moderate stringency wash (1 x SSC/0.1% SDS 2 x 30min at 55°C) and the other set was given a high stringency wash (0.1 x SSC/0.1% SDS 2 x 30min at 60°C) then dried. An autoradiograph was produced from the filters as described in Chapter 2, Section 6.6. The results are shown in Figure 5.3. These results are after a moderate stringency wash. Increasing the washing stringency reduced the intensity of the signal but did not change the pattern.

Clearly all three probes performed as expected with the positive and negative control RNAs (from BVDV-NADL infected and uninfected BT cells respectively).

Figure 5.3 Autoradiograph of dot blot hybridisations of clones 35, 50 and 75 to cellular RNA of infected cells.

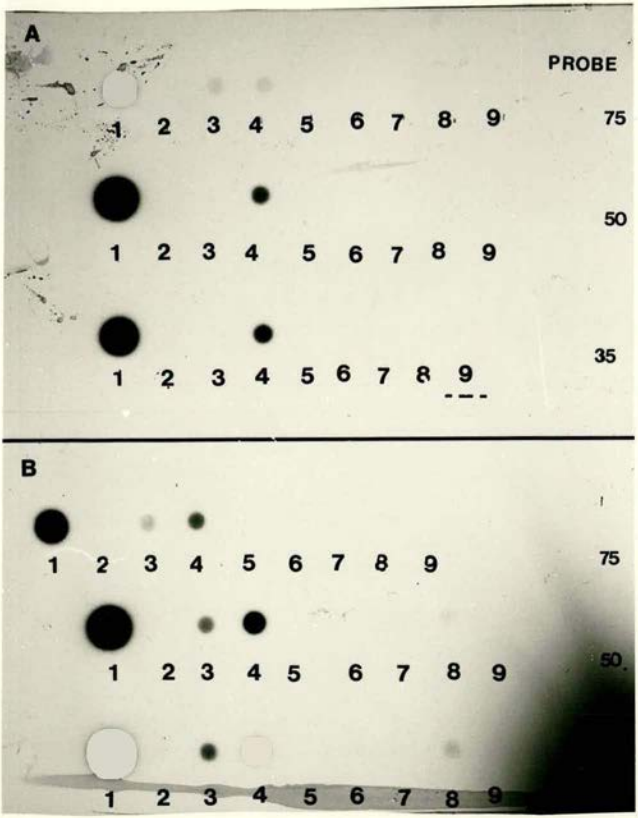
A Standard stringency: 50% formamide

B Low stringency: 25% formamide

RNA dots

1. NADL (BVD CP)
2. Osloss (BVD CP)
3. B816 (BVD CP)
4. 8085 (BVD CP)
5. BDV-M (CP)
6. G982 (BVDV NCP)
7. G1480 (BDV NCP)
8. G2048 (BDV NCP)
9. Uninfected FLM

Wash - 1 x SSC 42°, 2 x 30'


A
PROBE
1 2 3 4 5 6 7 8 9
75
50
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
35
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
75
1 2 3 4 5 6 7 8 9
50
1 2 3 4 5 6 7 8 9

At the lower (25%) formamide concentration all cytopathic BVDV strains except Osloss were recognised by all probes. The Osloss RNA had presumably been degraded. All three probes reacted more strongly with CP BVDV strain 8085 than with B816 implying the former is more similar to the NADL strain.

Only one of the three BDV strains tested showed a positive hybridisation result. The NCP strain G2048 reacted with probes 35 and 50, implying it is more similar to BVDV than are the other BDV strains. The signal was substantially weaker than with BVDV for both probes but it was impossible to determine if this was due to a difference in specificity or simply in amount of viral RNA present, since BDV grows to about 10-fold less than BVDV. The CP BDV strain showed no reaction with any of the probes, nor did the NCP strain G1480, which is the strain used in cDNA cloning work.

On raising the hybridisation stringency to standard conditions (50% formamide 42^{o}C) several positive reactions were lost. The reactions of probe 75 with the CP BVD strains were unchanged, but probes 35 and 50 no longer recognised the CP B816 strain, or any BDV strain except a weak signal between strain G2048 and clone 35.

In summary, at low hybridisation stringency all BVDV strains except Osloss were recognised by all three probes and one BDV strain was recognised by two of the probes (35 and 50). At higher (standard) stringency, probes 35 and 50 were almost NADL specific, showing hybridisation only to one other strain (8085). Probe 75, however, maintained a broader specifity, showing the same hybridisation pattern as at 25% formamide.

Therefore none of these probes was of potential use for detecting sequences of the BDV strain G1480 used throughout this project.

5.4.2 Clones p503 and pN24

Probes were produced from both clones as described in the previous section, and these probes were hybridised under standard conditions (42°C, 50% formamide) to dots of RNA from cells infected with various pestivirus strains. Both probes were tested against BVDV-NADL, BVDV Osloss, NCPBD strain G1480 and uninfected cell RNA. The results are shown in Figure 5.4, part A.

The washing conditions in this experiment were stringent: 0.1xSSC/0.1% SDS, 60°C for 2 x 30min washes. Under these conditions both probes pN24 and p503 were specific for the NADL strain of BVDV. A further experiment was done in which pN24 was hybridised under the same conditions to RNAs of a wider panel of virus strains and the filters washed under less stringent conditions (1 x SSC 0.1% SDS 42°C 2 x 30min). The results are shown in Figure 5.4, part B. Under these conditions pN24 recognises all the cytopathic pestivirus strains including BDV-CP. The strongest signals were obtained with strain C24V and BDV-CP. An interesting result is that strain 8085 (CP BVDV) reacted less strongly than BDV-CP with probe pN24, although in the previous experiment strain 8085 gave a very strong signal with probe 50 which is derived from sequences adjacent to those of pN24.

p503 was not tested under these less stringent washing conditions. In summary, probes pN24 and p503 are BVDV-NADL specific under standard hybridisation conditions followed by a high stringency wash. Probe pN24 recognised three CP viruses (two BVDV, one BDV) but no NCP viruses when the washing stringency was reduced.

5.4.3 RNA probes

Figures 5.5 and 5.6 show the results of hybridising two different RNA probes derived from pBV4/p80 to dots of RNA from a panel of pestivirus strains. In Figure 5.5 a short 192bp riboprobe from the downstream end of the p80 region was used. Hybridisation took place at 42°C 50% formamide and washing was

Figure 5.4 Autoradiograph showing dot blot hybridisation using clones pN24 and p503 to cellular RNA from infected cells

A
1. NADL
2. Osloss
3. BDNCP (G1480)
4. Uninfected FLM

B
1. C24V
2. BDNCP (G1480)
3. Osloss
4. NADL
5. Uninfected FLM
6. BDV-M (CP)
7. G2048
8. BVDV 8085

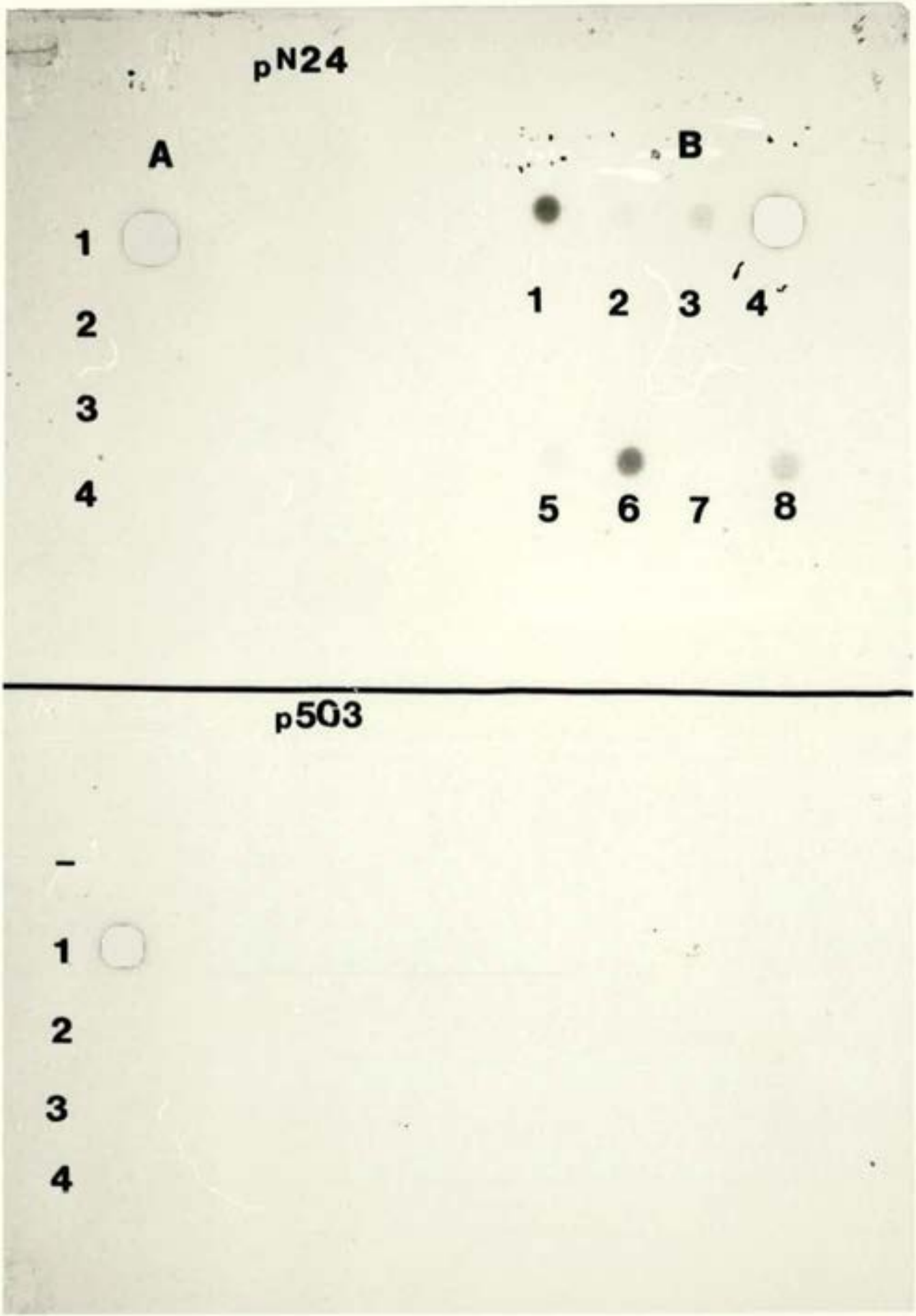
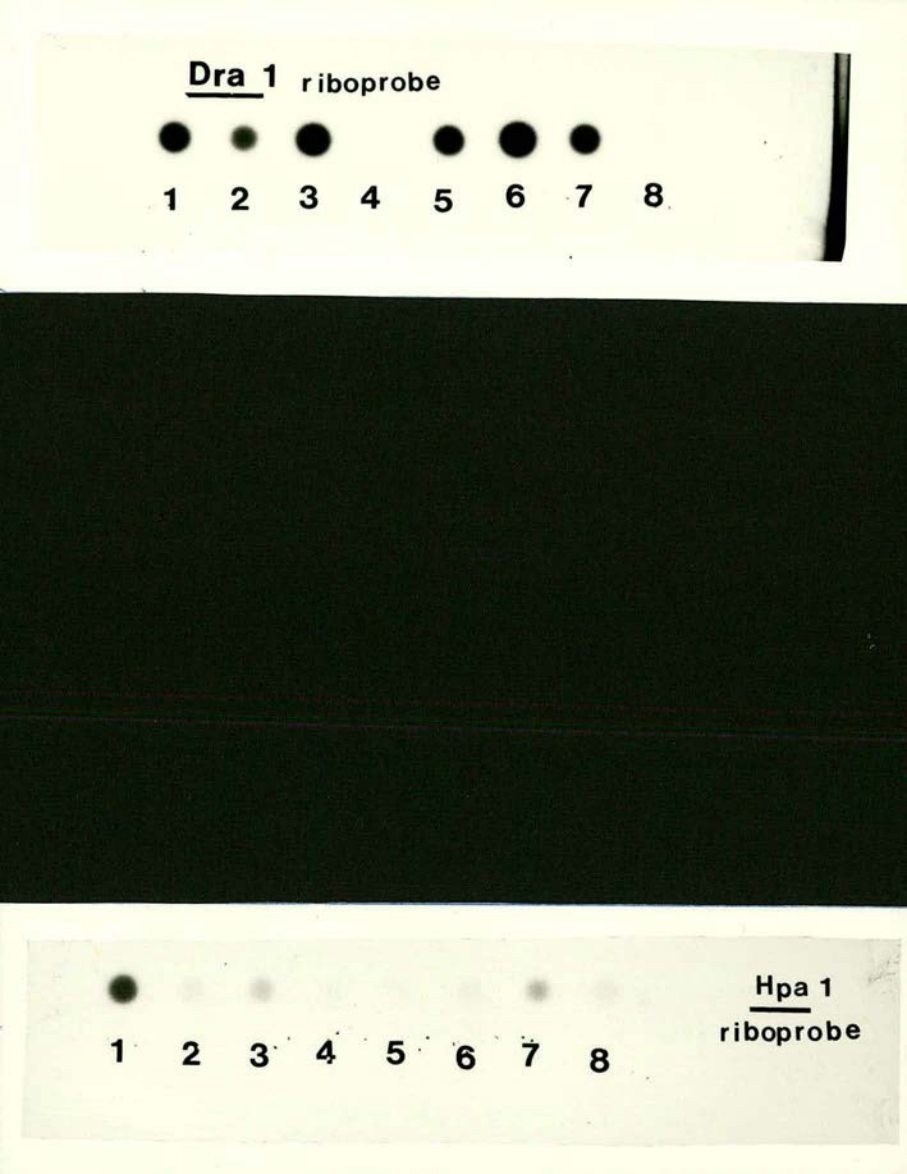

pN24
A
B
1
2
3
4
1
2
3
4
5
6
7
8
p503
–
1
2
3
4

Figure 5.5 Autoradiograph showing dot blot hybridisation to BVDV and BDV strains using the short (192bp) riboprobe produced by linearising pBV4/p80 with *Dra*I. Dots are total RNA from infected cells.

1. BVDV NADL
2. BVDV Osloss
3. BVDV C24V
4. BVDV G982
5. BDV G1480
6. BDV G2048
7. BDV M(CP)
8. Uninfected FLM cells

Figure 5.6 Autoradiograph showing dot blot hybridisation to BVDV and BDV strains using the long (2kb) riboprobe produced by linearising pBV4/p80 with *Hpa*I. Dots are cellular RNA from infected cells.

1. BVDV NADL
2. BVDV Osloss
3. " C24V
4. " G982
5. BDV G1480
6. BDV G2048
7. " M (CP)
8. Uninfected FLM cells


Dra 1 riboprobe
1 2 3 4 5 6 7 8
Hpa 1
riboprobe
1 2 3 4 5 6 7 8

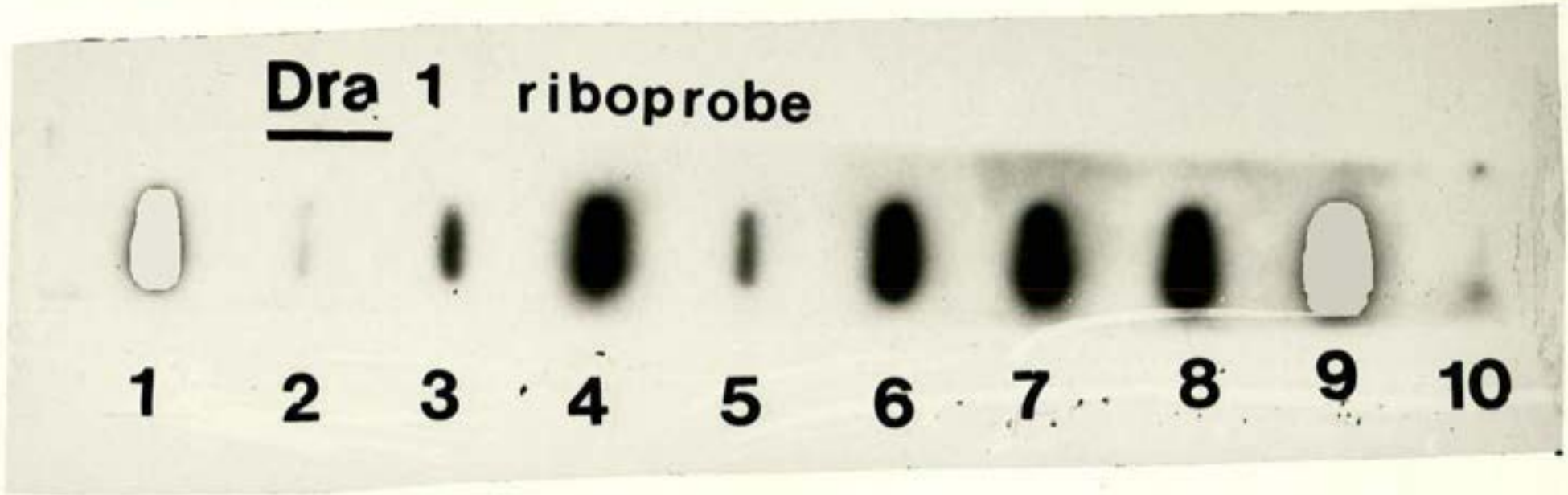
under low stringency conditions (1 x SSC 0.1% SDS at 42°C for 2 x 1hr washes). Higher stringency washes made the probes BVDV specific. At the lower stringency the *DraI* riboprobe appears to recognise all of the seven pestivirus strains tested, and gave as strong a signal with BD strains as with BVDV strains. (The lack of signal with NCP BVDV strain G982 was taken to be a false negative, due to lack of sample on the membrane, rather than a true result). Thus this short riboprobe looked extremely promising as a general pestivirus probe. When a longer probe which spanned the full length of the p80 insert was used, the specificity became much more rigid. This long riboprobe was hybridised and the filters washed under the same conditions as the short one, and the results are shown in Figure 5.6. Even under the low stringency washing conditions used, this probe recognised only the CP strains of virus (BVDV and BDV). (A positive result was taken as a signal which was greater in intensity than that of the negative control). Thus the increased length of this probe must have introduced areas of low homology between strains, whereas the short *DraI* riboprobe must represent a small highly conserved area.

In a further experiment, this short riboprobe was hybridised (under identical conditions) to an extended panel of BDV strains, results are shown in Figure 5.7. Only one strain of the nine tested failed to react (strain L991) strains D861 and R2727 showed a faint signal whereas the remaining six strains (all NCP) showed an equally strong signal.

In summary the short riboprobe derived from plasmid pBV4/p80 was shown to be specific over a broad range of BVDV and BDV strains making it potentially an extremely useful tool in the detection of pestivirus RNAs. The longer probe produced from the same clone had restricted specificity. Other lengths of riboprobe were not produced and tested, but it could be envisaged that a family of probes of differing lengths and specificities might be produced from this clone.

Figure 5.7 Autoradiograph showing dot blot hybridisation of the *Dra*1 riboprobe to various BDV stains shown. Dots are from BDV infected FLM cells.

1. JH2876
2. L991
3. D861
4. R2727
5. "Weybridge"
6. G2048
7. G1480
8. G1305
9. A1870
10. Uninfected FLM cells


Dra 1 riboprobe
1
2
3
4
5
6
7
8
9
10

5.4.4 Northern hybridisation using riboprobes from pBV4/p80

Having identified a probe which seemed potentially useful over a broad range of virus strains, the next stage was to use this probe in Northern blotting experiments to identify the size of RNA species to which it hybridised.

Samples of the same RNAs from infected cells as were used in the dot blot experiments were run on denaturing formaldehyde gels, the gels were blotted onto a nitrocellulose membrane and the blots probed with either the short or long pBV4/p80 riboprobe. Details of these procedures can be found in Chapter 2, Sections 5.2 and 6.2

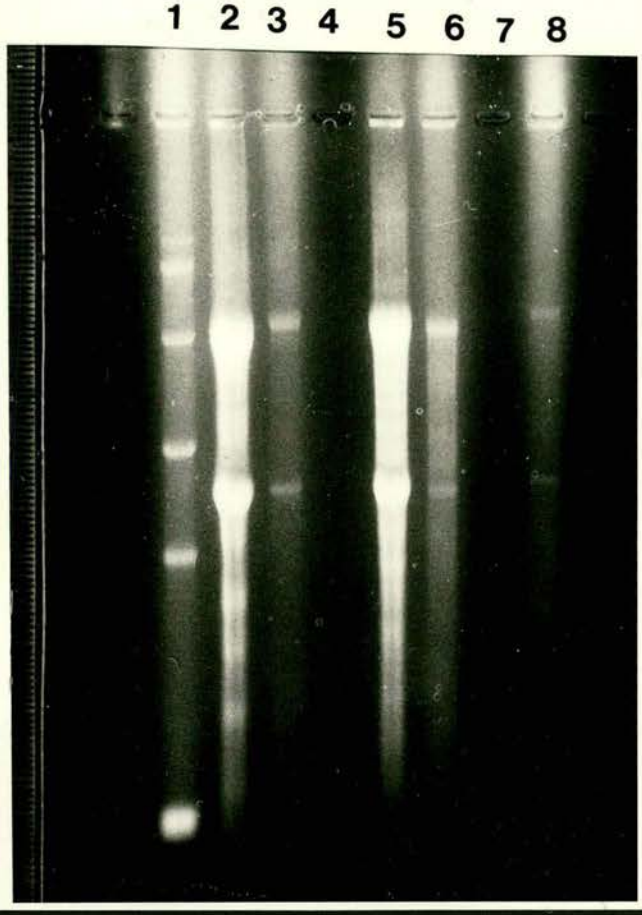
Figure 5.8A and B shows the results of a Northern blot of BVDV-NADL alongside two NCP BDV strains (G2048 and G1480). In A, the gel has been stained with ethidium bromide to show the marker RNAs and to check that the RNA samples were not degraded. In B the results are shown on an autoradiograph after hybridising the blot to the short *Dra1* riboprobe at 42°C in 50% formamide, and washing under stringent conditions (0.1 x SSC 0.1% SDS at 60°C for 2 x 20min). Under these conditions the probe hybridised to a single high molecular weight band in the NADL RNA sample and to no RNA species in any of the BD samples. The size of the high molecular weight band was approximately 12kb as expected for the BVDV genomic RNA. The lack of signal in the BDV samples was not due to lack of RNA, as can be seen in the UV photograph, the BDV tracks contain as much cellular RNA as the BVDV tracks. Nor is it likely to be due to a lack of virus RNA in the sample, since virus titres were reasonable (Table 5.3) and the same sample had given a positive dot blot result at a lower washing stringency. Therefore at the high washing stringency used, the *Dra1* riboprobe appears to be BVDV specific, the extent of this specificity between different BVDV strains was not tested on a Northern blot.

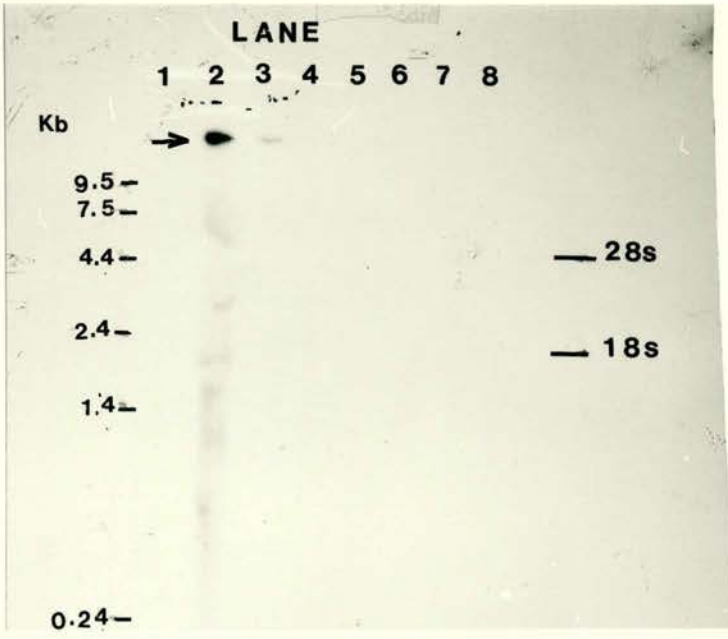
In the next experiment the above hybridisation using the short *Dra1* riboprobe was repeated on a Northern blot of various BVDV and BD RNAs but the washing stringency was reduced to 1 x SSC 0.1% SDS at 42°C 2 x 30min, in an attempt to detect

Figure 5.8 A Polaroid photograph of denaturing agarose gel containing cellular RNA from BVDV or BDV infected cells.

Track		
1.	RNA ladder	5. BDNCP G1480
2.	BVDV-NADL	6. BDNCP G2048
3.	NADL virion RNA*	7. -
4.	-	8. Uninfected FLM cells

B Autoradiograph showing results of Northern blot of the above gel, probed with the *Dra*1 riboprobe.


1 2 3 4 5 6 7 8


LANE
1 2 3 4 5 6 7 8
Kb
9.5
7.5
4.4
2.4
1.4
0.24
28s
18s

hybridisation of the probe to BDV RNA as in the dot blot results shown in Figure 5.7. The results of this hybridisation are shown in Figure 5.9. The decreased wash stringency allowed hybridisation of the probe to all RNAs present. High molecular weight RNA was visible in the BVD-NADL sample but not in any other strain. This result throws confusion on the positive dot blot results obtained with this probe under the same low stringency washing conditions - the negative controls are showing less of a signal than the infected samples in both cases, but the Northern blot reveals that the signal in the infected samples is due to hybridisation to RNA species whose size seems to indicate that they are of cellular and not viral origin except in the case of BVDV-NADL where an RNA of the correct size was detected.

In summary the *Dra*1 riboprobe was BVDV specific at high stringency and detected a 12kb band on Northern blots. At low stringency the specificity was blurred, all infected samples gave positive signals with low molecular weight RNAs but no high molecular weight bands were detected from any sample other than BVDV NADL.

Figure 5.10 A and B shows the results of a Northern blot of BVDV NADL and BDNCP (strain G1480) probed with the longer (more specific) *HpaI* riboprobe. The hybridisation and washing were carried out as in the previous experiment, ie standard hybridisation conditions followed by non-stringent washing of the blot. Since this probe appeared more specific it was hoped that this would eliminate the apparent non-specific hybridisation to cellular RNAs. However, as can be seen, this was not the case. At this stringency, the *HpaI* riboprobe gave exactly the same pattern as the *Dra*1 riboprobe, ie a strong positive signal from a high molecular weight RNA in the BVDV-NADL samples but non-specific hybridisation to 28s and 18s cellular ribosomal RNA in all other samples. This non-specific hybridisation was also evident in the NADL sample of RNA from infected cells, though not in the NADL sample RNA from purified virus, a further indication of the cellular origin of these lower molecular weight bands.

Figure 5.9 Autoradiograph showing results of Northern hybridisation experiment using the *Dra*1 riboprobe.

1. RNA ladder
2. BVDV NADL infected BT cell RNA
3. Uninfected BT cell RNA
4. BDV G2048 infected FLM cell RNA
5. Uninfected FLM cell RNA
6. BDV-M (CP) infected FLM cell RNA
7. BDV G1480 infected FLM cell RNA
8. BVD NCP (G982) infected BT cell RNA
9. BVD Osloss infected BT cell RNA

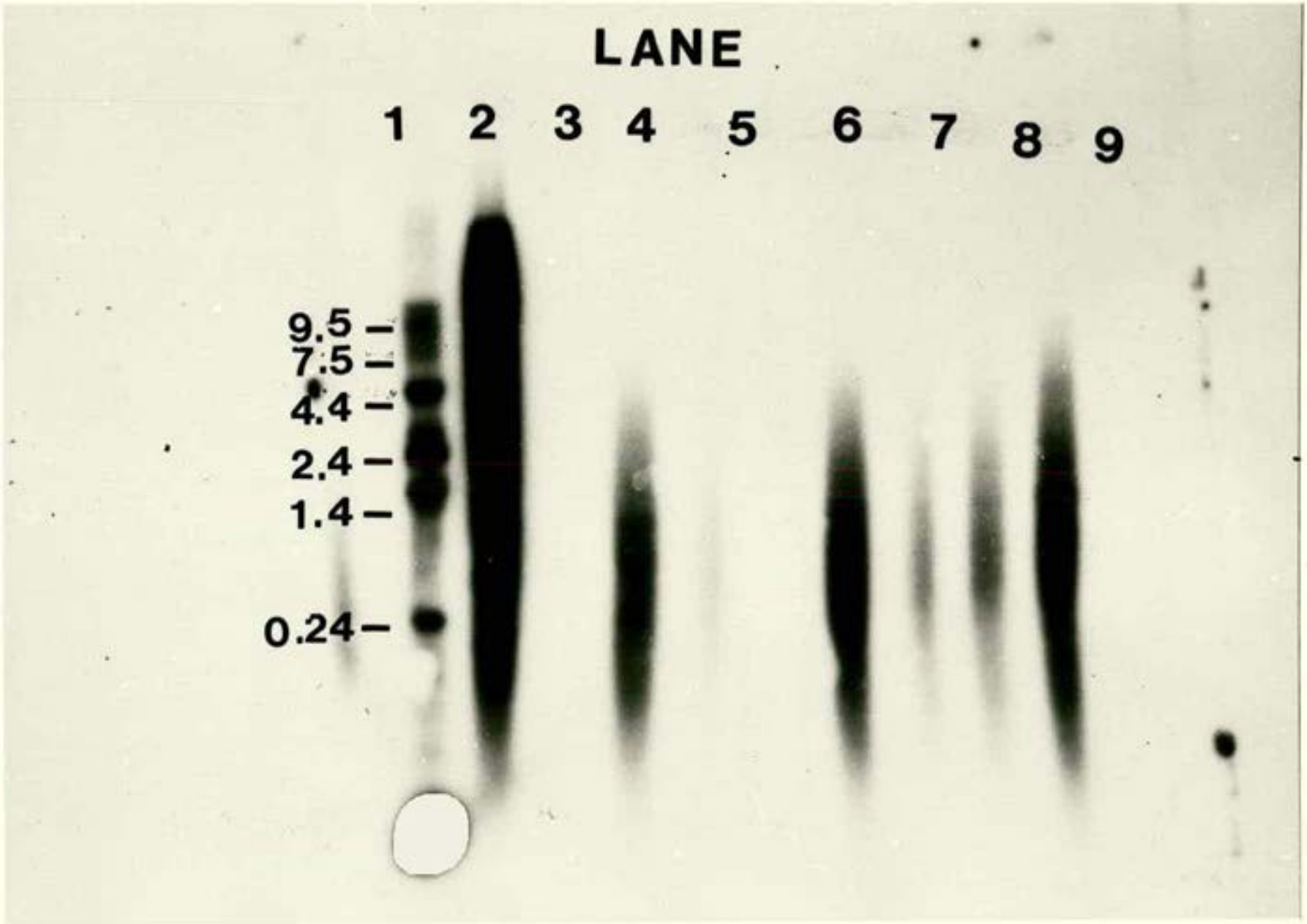

LANE
1 2 3 4 5 6 7 8 9
9.5
7.5
4.4
2.4
1.4
0.24

Figure 5.10 A Denaturing agarose gel stained with ethidium bromide, showing RNAs from infected and uninfected cells as detailed below.

1. Uninfected FLM cell RNA

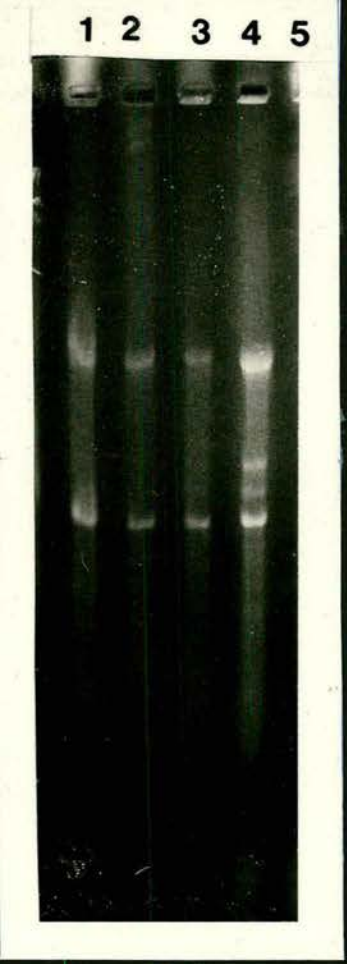
2. Uninfected BT cell RNA

3. BDNCP (G1480) infected FLM cell RNA

4. BVDV-NADL infected BT cell RNA

5. BVDV-NADL RNA from purified virus

B Autoradiograph of a Northern blot of the above gel probed with the HpaI riboprobe


1 2 3 4 5


LANE
1 2 3 4 5
28s
18s

In summary, the short *Dra*1 riboprobe was used at high stringency to demonstrate the 12kb BVDV-NADL genomic RNA on Northern blot, but failed to detect RNA from BDV (NCP strains). At lower stringency the positive results obtained on dot blots with both short and long riboprobes were shown probably to be due to hybridisation of these probes to the ribosomal RNAs of infected cells as detected on Northern blots, or to mRNA species induced by infections.

5.5 SUMMARY

Six BVDV clones were used to produce probes which were tested for their hybridisation to various BVDV and BDV strains. Few positive signals were obtained with any BDV samples although a range of BVDV strains were positive. The BDV hybridisation results could be summarised as follows: Clones 35 and 50 detected BDNCP G2048, Clone pN24 detected cytopathic BDV, and the riboprobes derived from pBV4/p80 detected various BDV strains on dot blots but failed to reveal high molecular weight BDV RNA on Northern blots. Thus no probe was obtained with which to screen the possible cDNA library produced from the BDNCP strain G1480 used in this project.

This implies that at least over the areas tested nucleic acid homology between BVDV and BDV is quite low.

CHAPTER 6

THE POLYMERASE CHAIN REACTION

6.1 INTRODUCTION

The polymerase chain reaction (PCR) is an *in vitro* method for primer-directed amplification of specific DNA sequences. Originally developed by Saiki *et al* (1985), it was designed to provide highly efficient amplification and purification of DNA sequences of interest.

PCR relies on the availability of sequence information for sites that flank the region to be amplified. Two short synthetic oligonucleotides in opposing orientations are prepared as primers, complementary to each of these sites. Reiterative cycles of DNA denaturation, primer annealing and primer extension are then used to generate multiple copies of the primer-flanked target sequence. The efficiency and sensitivity of the technique is unparalleled, a million copies of a target can be produced in a few hours, and amplification can be achieved from one target molecule in a single cell (Li *et al*, 1990). Although originally developed for use with target DNA sequences, PCR has been used successfully in conjunction with reverse transcription to amplify rare RNA sequences (Doherty *et al*, 1989). PCR has also been used to detect and analyse viral sequences in infected cells, for both DNA and RNA viruses (Kwok *et al*, 1987, Gama *et al*, 1988). There is one report in the literature describing the use of PCR to amplify sequences from the BVDV genomic RNA (Schroeder and Balassu-Chan, 1990).

PCR is of great potential value in pestivirus research for several reasons. Current diagnostic methods for the detection of pestiviruses rely either on virus isolation or immunoassays which lack the sensitivity of PCR. PCR is also rapid and can be performed on very small amounts of material, amplification is readily achieved on RNA prepared from lymphocytes in clinical material. It is also an excellent way to detect non-cytopathic pestiviruses which can be difficult to detect by other diagnostic techniques.

This chapter describes the use of PCR to amplify various pestivirus sequences as follows: Two complete sequences of different BVDV strains (Osloss and NADL) were available. Based on sequence comparisons (for further discussion on sequence comparison see Chapter 7) conserved areas of the BVDV genome were identified and used to design complementary primers, covering various parts of the genome. These primers were tested firstly on the NADL strain of BVDV, and then on other BVDV and BD strains.

To perform PCR on pestiviruses, an initial reverse transcription step to produce cDNA is necessary. This cDNA could be primed using random hexamers as in Chapter 4, or else by using the specific anti-sense oligonucleotide primer. Both approaches were tried. Once the amplification of cDNA had been achieved, the amplified sequences could be used as probes for the detection of pestivirus RNA or screening a cDNA library.

6.2 DESIGN OF PRIMERS FOR PCR ON PESTIVIRUSES

The design of primers is the first step in any PCR experiment and three factors need consideration. Firstly, where to position the primers; this depends on where the areas of interest occur, the desired length of amplimer and the position of conserved stretches of sequence to be used as primers. Secondly, the actual sequence of the primers, which may not always be a straightforward complement of the target strand, if a broader specificity is required. Lastly, the physical properties of the primer, such as length and G/C content, are also important.

In this work five sets of primer pairs were tested in PCR reactions. Two of these pairs were kindly supplied by M. Sweeney, University of Warwick, and the other three were designed specifically. A summary of the primers can be found in Figure 6.1 and Table 6.1.

Primer pair $325_C/326_C$

These span a target sequence 299bp in length covering most of the 5' untranslated region of the genome. This region is highly

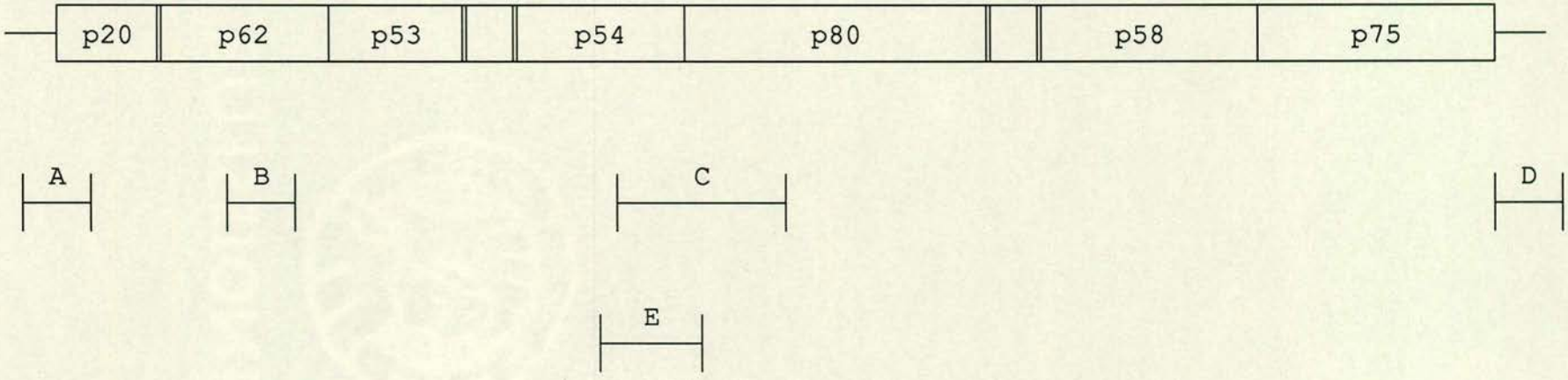
TABLE 6.1

Oligonucleotide	Sequence (5' ——> 3')	Length (bp)	G/C Content	Position on NADL RNA
325_C	AGAGCCTAGCCATGCCCTTAGT	22	54%	97 - 118
326_C	TCAACTCCATGTGCCATGTAC	21	48%	395 - 375
NADL2	CTTGTTGACGGGTTGAC	17	53%	1739 - 1755
$NADL2_R$	GGTGCCAAATTTCCCAGG	18	55%	1994 - 1977
NADL6	GAAAGCATTATGAGCAACT	19	37%	12204 - 12222
$NADL6_R$	TACAGCTAAAGTGGTGTGTGC	21	48%	12523 - 12503
718_D	CCGCTAGGGGGCAA*TATTTC	21	52%	5301 - 5321
719_D	TGCTG*CGTGG*AGTCAT	18	50%	6484 - 6467
928_G	GTTTAGTGGCAGCACTCATAGAGCTGAACT	30	46%	4614 - 4643
929_G	TAAGCTCGTAGGGAACCTCACCGGGGCTCT	30	60%	5557 - 5529

* Denotes inosine which has non-stringent pairing ability

Figure 6.1 Relative positions of primer pairs used in PCR on the BVDV-NADL genome. Not to scale.

BVDV GENOMIC RNA



PRIMER PAIRS

A	325_C / 326_C	D	NADL6 / 6_R
B	NADL2 / 2_R	E	928_G / 929_G
C	718_D / 719_D		

conserved, at around 90%, between NADL and Osloss BVDV strains, as would be expected, since the control and recognition sequences for virus gene expression are likely to occur in this region. Thus it would be reasonable to expect these primers to have a broad range of specificity. Additionally, the resulting amplified sequence should be useful as a general probe for the pestiviruses.

Primer pair NADL 6/NADL 6_R

These span a target sequence 320bp long covering the last 100 bases of the p75 coding region and stretching into the 3' untranslated region. The sequence divergence in this region is around 25% mismatch, although this is not an even distribution, there are very highly conserved regions interspersed with variable regions. The 3' untranslated region must contain recognition sequences for the viral RNA polymerase, and there are other conserved areas in this region. In particular, there are sets of repeated sequences which are conserved between the NADL and Osloss strains (see Chapter 7). This target sequence then, is also likely to have a broad specificity.

Primer pair NADL 2/NADL 2_R

These primers flank a region 256bp long in the area coding for the p62 glycoprotein. They were derived solely from the sequence of the NADL strain and should therefore be specific for this strain, since their sequences are not conserved in the Osloss strain. The target sequence amplified by these primers should have some discriminatory uses since there is considerable inter-strain variation in this glycoprotein region (71% homology between NADL and Osloss strains).

Primer pair $718_D/719_D$

These primers flank a sequence of 1184bp in a region of considerable interest; the junction between p54 and p80 which is cleaved in cytopathic pestivirus strains and not in non-cytopathic strains. The p54 sequences which make up roughly half of the target sequence amplified by these primers are highly variable. This region includes the insertion of ubiquitin

sequences which occur only in the Osloss strain. However, the p80 sequences are highly conserved - this region is the most highly conserved of the entire genome. It would be of great interest to use PCR of this region to define differences in the sequence of the p54/p80 cleavage site in different virus strains. For this reason, primers were needed which would anneal to a very broad range of pestivirus strains. Since sequences were only available for two BVDV strains, flavivirus sequences were searched for homologies with these existing BVDV sequences, with the rationale that any such homologies would be conserved between most, if not all, pestiviruses. The primer 719_D is positioned at a region where there is homology with flaviviruses at the protein level.

Primer pair $928_G/929_G$

These primers flank a 944bp region of the p54 coding sequence, which stops just prior to the p54/p80 processing point, and which actually overlaps the $718_D/719_D$ amplimer, so in effect it is a 5′ portion of the larger $718_D/719_D$ target sequence. Again this area is highly variable and includes the Osloss ubiquitin insertion. The sequences of these primers were kindly recommended by L. Moerlooze, who had achieved success in amplifying a cytopathic strain of BDV using them (Moerlooze *et al*, 1990).

6.3 EXPERIMENTAL

6.3.1 Preparation of stock solutions

Because of the extreme sensitivity of the PCR technique, several precautions were routinely taken to minimise the chance of amplification of contaminating DNA sequences.

All PCR work was performed wearing gloves, and the assembly and analysis of the reactions were carried out using separate sets of pipettes. Reagents were prepared in a sterile laminar flow hood, sterilised and aliquoted in small volumes. Aliquots of stocks were stored at -20°C.

Stock solutions

Buffers: 5 x MMLV reverse transcriptase buffer. Supplied with enzyme (BRL).

10 x PCR amplification buffer	500mM KCl
	100mM Tris Cl pH8.3
	15mM $MgCl_2$
	0.1mg/ml Gelatin

Buffers were prepared using sterile frozen stock solutions as described below.

dNTPs

dNTPs were used in excess: a final concentration of 200uM in the reaction. A stock solution containing 10mM of each dNTP was prepared by dilution of commercially available 100mM stocks (Pharmacia) in sterile water.

Oligonucleotide primers

These were used at 1uM in the reaction. Because of the differing lengths of primers and different supply concentrations the dilution factor for each primer as supplied was different. Table 6.2 shows the dilution of primers used.

Taq Polymerase

The enzyme used was "Amplitaq" supplied by Perkin Elmer Cetus. It was used at 5 units per 100ul PCR.

Table 6.2 Dilution of oligonucleotide stocks

Oligo	Length (bp)	1 uMolar	Supplied stock concentration	Required concentration (20x)	Dilution of stock required to give 20x
325_C	22	6.6ug/ml	320ug/ml	132ug/ml	1/2.5
326_C	21	6.9ug/ml	320ug/ml	138ug/ml	1/2.5
NADL2	17	5.9ug/ml	500ug/ml	118ug/ml	1/4
$NADL2_R$	18	6.2ug/ml	500ug/ml	124ug/ml	1/4
NADL6	19	6.2ug/ml	500ug/ml	124ug/ml	1/4
$NADL6_R$	21	6.9ug/ml	500ug/ml	138ug/ml	1/3.5
718_D	21	6.9ug/ml	220ug/ml	138ug/ml	1/1.5
719_D	18	5.9ug/ml	280ug/ml	118ug/ml	1/2.5
928_D	30	9.8ug/ml	400ug/ml	198ug/ml	1/2
929_D	30	8.9ug/ml	300ug/ml	178ug/ml	1/2

6.4 METHODS

The overall strategy for the PCR performed on pestiviruses was to use RNA prepared from virus-infected cells as the input source of target, to prepare cDNA from this RNA and then directly amplify this cDNA in a PCR. The cDNA reaction was diluted into PCR buffer thereby allowing a one tube reaction which minimises losses.

RNA isolation

RNA was prepared from virus infected cells using the guanidinium isothiocyanate method (Chapter 2, Section 4.5) and pelleted through a 5.7M CsCl cushion. RNA was routinely prepared from two 50ml cultures of monolayered cells, harvested before CPE had become pronounced. This RNA was stored under ethanol at -20°C, and aliquots removed for PCR as required.

cDNA synthesis

The cDNA reactions were standard for all PCR experiments and were performed in a 20ul volume. The RNA was mixed with either random hexamers at 10ug/ml or the specific downstream oligonucleotide primer at 25ug/ml (final concentrations). The reaction was heated to 96°C for 3 min then cooled to 37°C. The rest of the reaction components were added as follows:

4ul MMLV reverse transcriptase buffer
1ul 10mM dNTP stock
1ul RNA guard (Pharmacia)
1ul MMLV reverse transcriptase*

* This was the cloned enzyme lacking the RNAse H activity supplied by BRL. It was vastly superior to the AMV enzyme (Anglian Biotech) in producing cDNA from unpurified RNA populations (results not shown).

The cDNA reaction was allowed to proceed at 42°C for 30min.

Amplification of first-strand cDNA

The 20ul first-strand cDNA reaction was diluted to 100ul volume as follows:

60ul H_2O
5ul each oligonucleotide primer (20 x stock)
10ul 10 x PCR buffer

This mixture was overlaid with 100ul light mineral oil, boiled for 3min, then chilled on ice. The rest of the components were added (through the mineral oil):

2ul 10mM dNTPs
0.5ul (5 units) Taq polymerase

The reaction was amplified through the desired cycles.

Amplification Cycles

The standard cycle used was: 95°C for 90s denaturation
37°C for 2min annealing
72°C for 3min extension

Generally 25-30 cycles were performed
The cycles used were varied as required.

Once amplified, reactions were frozen at -20°C and the mineral oil removed. A sample of the reaction (one fifth - one tenth of the volume) was analysed on an agarose gel by ethidium bromide staining.

6.5 RESULTS

In this work five primer pairs were tested for their ability to prime amplification on target cDNA prepared from cells infected with BVDV and BDV.

6.5.1 Primers $325_C/326_C$

These were firstly tested on RNA from BVDV NADL infected cells.

cDNA synthesis was performed using both random priming and specific priming by oligo 326_C. The cDNA was amplified by PCR by the standard method (see previous section) through 30 cycles. One fifth of the reaction volume was run on a 1.5% agarose gel and stained with ethidium bromide. The results are shown in Figure 6.2

Figure 6.2 Agarose gel (2%) showing PCR result using primers $325_C/326_C$ on NADL-infected BT cell RNA

Track: 1 1kb ladder

2 1st strand primed by 326_C

3 1st strand primed by random hexanucleotides

4 As track 2

5 1/20th of the reaction in track 2

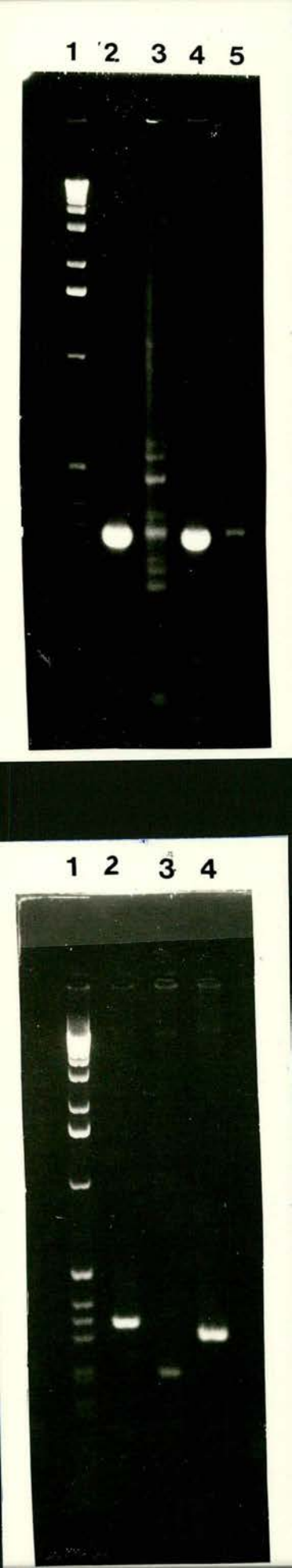
Figure 6.3 Agarose gel (2%) showing PCR result using 3 different primer sets on NADL-infected BT cell RNA

Track: 1 1kb ladder

2 NADL6/$NADL6_R$ primers

3 NADL2/$NADL2_R$ primers

4 $325_C/326_C$ primers


1 2 3 4 5
1 2 3 4

The expected amplimer size was approximately 300bp. Track 2 shows the result with specific priming and track 3 with random priming (Track 4 is the same as track 1 and track 5 is one twentieth of the reaction volume loaded).

A cleanly amplified fragment of the expected size was obtained from BVD-NADL sequences using PCR amplification of cDNA primed by oligo 326_C. Amplification of random primed cDNA gave a fragment of the same size but a "ladder effect" was obtained due to non-specific amplification of other sequences.

Thus, under standard conditions, 30 cycles of amplification using primers $325_C/326_C$ produced the expected 300bp amplified fragment from BVD-NADL RNA.

6.5.2 Primers NADL $2/2_R$ and NADL $6/6_R$

In the next experiment, shown in Figure 6.3, two more primer pairs were tested on the same BVD-NADL RNA, again under the same conditions as above. Track 2 shows amplification by primers NADL $6/6_R$, track 3 by primers NADL $2/2_R$ and track 4 by primers $325_C/326_C$. The expected amplimer sizes were 320bp, 257bp and 298 bp respectively. One tenth of each reaction was run on the gel, which was 1.5% agarose.

Successful amplification was achieved using all three primer sets after 30 cycles. The NADL $2/2_R$ pair appeared least efficient, the other two pairs seemed to be of equal efficiency. The next stage was to try these two most efficient primers on BDV RNA. In any case the NADL $2/2_R$ primers would be least likely of these three sets to prime on BDV RNA, since they are likely to be the most specific, being derived from a variable region of the genome (Figure 6.1).

Figure 6.4 shows the results of amplification performed on oligo-primed cDNA from the RNA of BVDV-NADL and BDV-G1480 (NCP) infected cells. Both primer pairs gave successful amplification on the positive control RNA (BVDV-NADL) but unfortunately the resulting bands were very faint. Neither pair primed amplification on the BDV sample.

Varying conditions described below were used in an attempt to achieve amplification on the BDV RNA template. The following changes were made to the standard protocol:
(a) 35 cyles were used as opposed to 30, in case the BDV samples had lower numbers of target molecules. This had no effect on amplification of BDV sequences (result not shown).
(b) The already non stringent annealing temperature of 37°C was decreased to room temperature to see if this allowed primer annealing and extension on the BDV samples. This, too, was unsuccessful (result not shown). This result implies that the problem is not due to poor primer annealing, since under such non stringent conditions it is unlikely that the primers would not anneal especially since these primers are presumed to be conserved. The problem appears then to be at the extension rather than the annealing stage. If there is a mismatch in base pairing at or near the priming end of the oligonucleotide, priming will not occur even if the rest of the primer is tightly bound. It seems to be the case for these primer pairs that one or both of the pairs may have such a mismatch with the BDV sequence at their priming ends.

These primers did not seem to be of use as a route to amplify BDV sequences. At this stage it was decided to design another set of primers derived from the most conserved region in BVDV, the p80 coding sequence. These primers, 718_D and 719_D, have been discussed fully in Section 6.2

6.5.3 Primers 718_D, 1719_D

Primers 718_D and 719_D were used in a PCR on BDV-infected cell RNA as follows: Primer 719_D was used to specifically prime first strand cDNA as discussed in Section 4. This cDNA was included in a PCR using this primer pair. The standard amplification cycle was adjusted to allow a longer extension time since the target sequence was 1.2kb long. Amplification was performed using 35 cycles of 95°C for 90S, 37°C for 2min and 72°C for 5min. 1/10 of the reaction was run on a 1.2% agarose gel. The results are shown in Figure 6.5. Tracks 2 and 4 are negative control samples from uninfected cells, track 3 is BDV

Figure 6.4 1% Agarose gel showing PCR on BVDV and BDV infected cell RNAs using primer pairs $325_C/326_C$ and $NADL6/6_R$.

1 1kb ladder

2 NADL infected BT cells ; $325_C/326_C$

3 NADL infected BT cells + NADL $6/6_R$

4 BDV infected FLM cells + $325_C/326_C$

5 BDV infected FLM cells + $325_C/326_C$

Figure 6.5 1% Agarose gel showing results of PCR using primer pair $718_D/719_D$ on RNAs from BVDV infected, BDV infected and uninfected cells.

1 1kb ladder

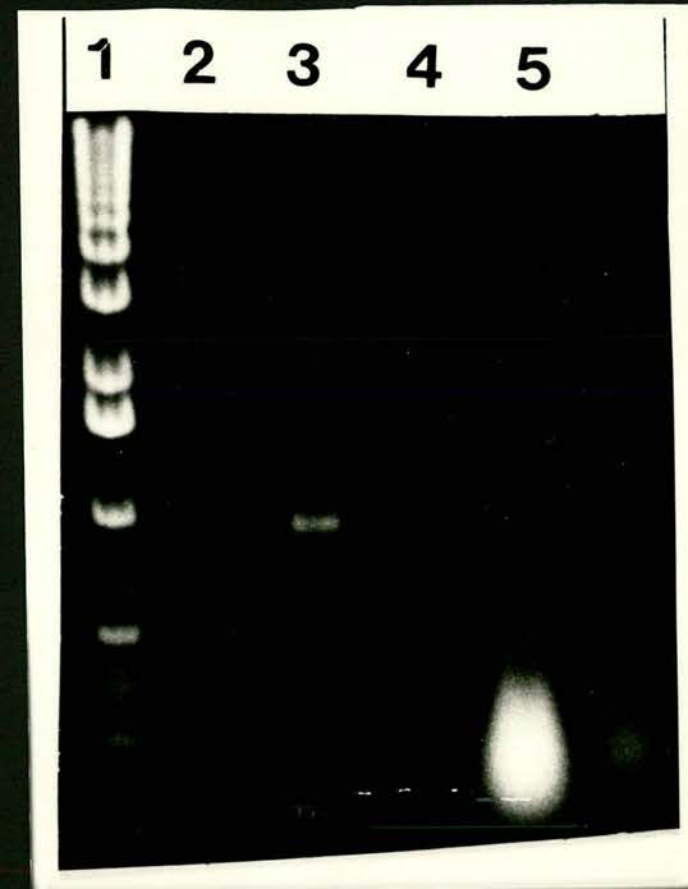
2 Uninfected FLM cells

3 BDNCP infected FLM cells

4 Uninfected BT cells

5 NADL infected BT cells


1 2 3 4 5


1 2 3 4 5

and track 5 is the BVDV-NADL sample. Thus the 718_D and 719_D primers appear to allow successful amplification on BDV RNA, but not on BVDV-NADL RNA. This is unexpected, since they were derived from the published NADL sequence.

Figure 6.6 shows a repeat of this experiment, this time including a positive control using primers $325_C/326_C$ on the NADL sample to check that the NADL RNA is viable. This figure shows that amplification is successful with the primers $325_C/326_C$ on NADL RNA (track 2), but unsuccessful with primers $718_D/719_D$ (track 3). Track 4 shows the successful amplification of BDV sequences using primers $718_D/719_D$. These primers failed to produce specific amplification on the negative control (uninfected cell) RNA. Thus primers $718_D/719_D$ are extremely valuable as a route to BD sequences from this area of the genome. The failure of these primers on a BVDV NADL template is difficult to explain, one explanation may be that the NADL RNA sample was degraded such that the $325_C/326_C$ control amplimer remained intact, but the $718_D/719_D$ sequence was destroyed. Another explanation could be that secondary structures in the NADL RNA prevented amplification over a length of 1.2kb, though this does not occur in BDV.

6.5.4 Primers $928_G/929_G$

These primers were designed on the basis of unpublished observations that they gave successful amplification with RNA from cells infected with the Moredun cytopathic strain of BDV (L. Moerloose, personal communication). However, in our hands they did not achieve successful amplification on either CP or NCP BDV strains, though they did give amplification on BVDV-NADL. Figures 6.7 and 6.8 show this. Amplification was performed under standard conditions by the methods described in Section 4, except that the extension time was increased to 4 min and the number of cycles was 35. Figure 6.7 shows PCR performed solely on a NADL sample: track 2 is the positive control with primers $325_C/326_C$, track 3 is an unsuccessful $718_D/719_D$ reaction, track 4 shows a positive result after

Figure 6.6 1% Agarose gel showing PCR result using primer pair $718_D/719_D$ on NADL and BDNCP infected cell RNA.

Track: 1 1kb ladder

2 $325_C/326_C$ primers on NADL (+ve control)

3 $718_D/719_D$ on NADL

4 $718_D/719_D$ on BDNCP

5 $718_D/719_D$ on uninfected FLM cell RNA

Figure 6.7 1% Agarose gel showing PCR result using three different primer pairs on BVDV NADL infected cell RNA.

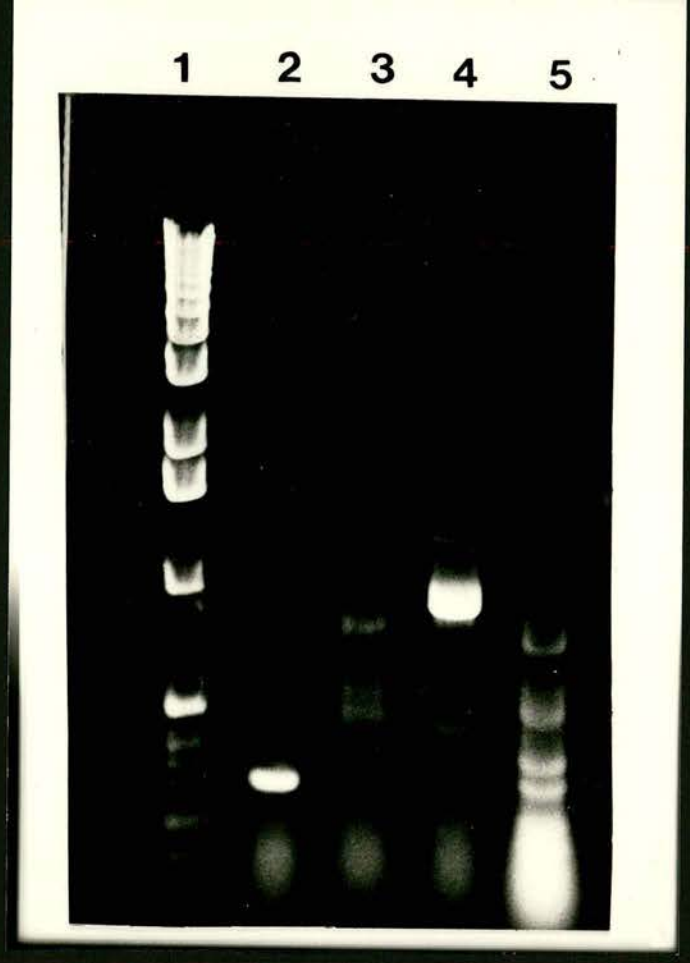
Track: 1 1kb ladder

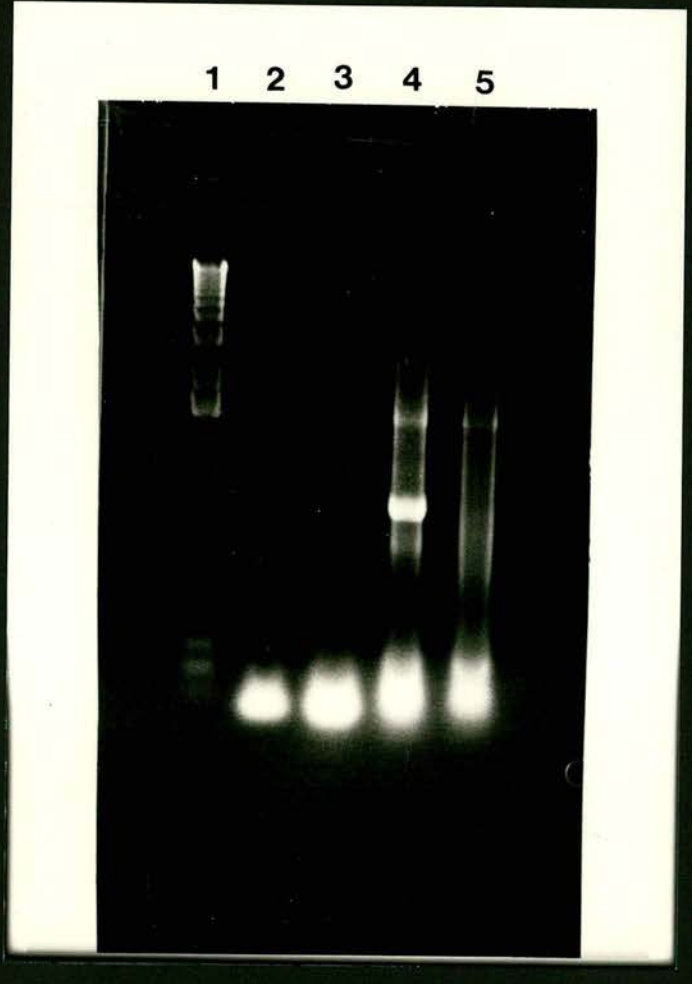
2 $325_C/326_C$ primers

3 $718_D/719_D$ primers

4 $928_G/929_G$ primers

5 $928_G/929_G$ primers on uninfected cell RNA


1 2 3 4 5


1 2 3 4 5

amplification using primers $928_G/929_G$, and track 5 shows a negative control sample of uninfected BT cells.

Figure 6.8 shows the results on BDV samples. Track 2 is a negative control using uninfected cell RNA, track 3 is a $718_D/719_D$ reaction on NADL RNA, track 4 is the positive control using primers 928_G and 929_G on NADL, and tracks 5 and 6 show the same primers used on BDNCP and BDCP respectively. Successful amplification was not achieved using either of the BD template RNAs. This result was repeated (not shown) altering the number of cycles, the magnesium concentration and the annealing temperature all had no effect (not shown) and NCP strains of BDV respectively, neither of which gave successful amplification using these primers.

6.6 CONCLUSIONS

Five oligonucleotide pairs were tested for their ability to prime amplification of BVDV and BDV sequences in PCR. Of these pairs, four produced successful amplification from BVDV-NADL and one from BDV-G1480.

It was found that the cDNA synthesis preceding amplification was best performed using specific priming from the downstream oligonucleotide, rather than using random hexamers. The amplifications were specific, particularly considering the relatively low (37^oC) annealing temperature used throughout.

An unexpected result was the lack of amplification on the BDV template using primers $325_C/326_C$ and NADL $6/6_R$. Both these pairs are from sequences which occur in the non-coding regions of BVDV and are very highly conserved. Lack of amplification using these primers was perhaps due to a mismatch at the priming end of one or both primers. Primer pair $928_G/929_G$ also failed to amplify BDV sequences. These primers had been previously shown to do so, the reason for their failure in our hands remains unclear.

Figure 6.8 1% Agarose gel showing PCR result of using different primer pairs on BDNCP and BDCP infected cell RNAs.

Track: 1 1kb ladder

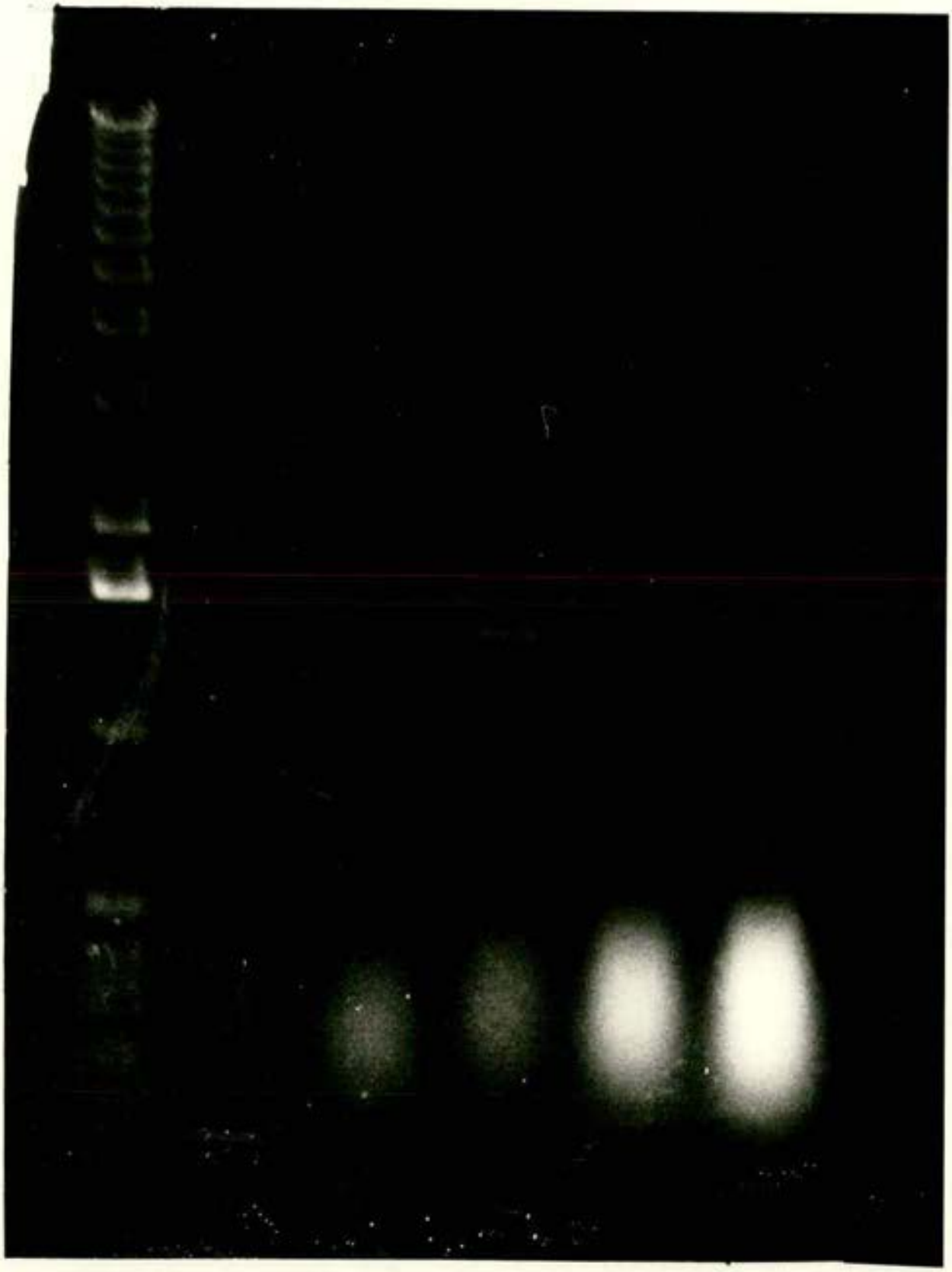
2 primers $928_G/929_G$ on uninfected cell RNA

3 primers $718_D/719_D$ on BVD NADL

4 primers $928_G/929_G$ on BVD NADL

5 primers $928_G/929_G$ on BDCP

6 primers $928_G/929_G$ in BDNCP


1
2
3
4
5
6

Primers $718_D/719_D$ were the only pair to successfully amplify BDV sequences. These produced a specific amplification of a 1.2kb fragment. Unfortunately, time did not permit verification of this result by hybridisation of the amplimer to BDV RNA.

The successful amplification of BDV sequences is a potentially valuable route to obtaining sequence information about the virus. The amplified fragment could be used as a probe to detect different pestivirus strains, as a primer for further direct PCR on BD, or as a probe for any cDNA library. In addition, amplification of this particular area of the genome is of interest, since it spans the processing junction of p54/p80. Unfortunately, because of limited time, these routes were not exploited.

Another potential experiment would be to test the four BVDV amplified sequences for their ability to hybridise to BDV-RNA in blots. An estimate of the homology between the different areas of the genome represented could be obtained in this way.

Lastly, the failure of four of the primer pairs to amplify BDV sequences may be due to a failure of one, rather than both, oligonucleotides. Different combinations of primers could be tried to see if this was the case, for example NADL 2_R with 325_C could potentially produce an amplified sequence 1900bp long encompassing the p20 and p62 coding sequences.

Chapter 7

COMPUTER ASSISTED SEQUENCE ANALYSIS OF PESTIVIRUSES

7.1 INTRODUCTION

At the outset of this project there was sequence information available for only one pestivirus: BVDV Osloss (Renard *et al* 1987c). Soon after, this was followed by the publication of the sequence of the RNA of BVDV-NADL (Collett *et al*, 1988c). The availability of two full-length BVDV sequences allowed a comparison to be made at both the amino acid and nucleotide levels.

Recently two reports describing the nucleotide sequence of HCV strains, Alfort and Brescia, were published (Moorman *et al*, 1990) (Meyers *et al*, 1989). This allowed further comparisons between pestivirus sequences.

Reports describing the comparison of pestivirus sequences have now been published (Moorman *et al*, 1990; Collett *et al*, 1988a). The comparisons undertaken in this thesis agree with the findings of these authors.

The most extensively studied pestivirus was BVDV NADL, for which many viral proteins have been mapped (Collett *et al*, 1988b), therefore comparisons were made with reference to this strain.

Sequence comparisons were undertaken primarily to find conserved stretches of nucleic acid sequence which would be useful as probes or primers for use in PCR.

Hardware and software

Computer comparison programmes were run on the AFRC Harpenden VAX system. The programs used were the University of Wisconsin package for analysis of sequence data (Devereux *et al*, 1984).

Sequence data

The BVDV-Osloss sequence was entered into a file directly from the published sequence, by M. Sweeney, University of Warwick, who kindly supplied us with a copy of the sequence on disk.

The BVDV NADL sequence was obtained on disk from M.S. Collett, Molecular Genetics Inc, Minnesota, USA. The HCV (Brescia) sequence was obtained on disk from R. Moorman, CVL, The Netherlands.

All sequences were transferred from disk to files in the VAX system for analysis.

7.2 ANALYSIS AND COMPARISON OF TWO BVDV SEQUENCES

7.2.1 Identifying open reading frames

Both NADL and Osloss sequences were searched for open reading frames (ORFs). The program "FRAMES" would have been ideal for this but it could not be used due to our lack of graphics facilities. Instead the program "MAP" was used. This programme finds and translates all reading frames on the + and - strands, as well as displaying restriction enzyme sites on the sequences covered. The option to translate only open reading frames from the + strand was chosen. This revealed one major ORF in the NADL sequence and two major ORFs in the Osloss sequence. The NADL ORF ran from position 386 to position 12352, and the Osloss ORFs from positions 384 to 4244 and 4396 to 12494. There are also several minor ORFs in both NADL and Osloss sequences.

Since one long ORF is present in NADL and two in Osloss the expression of proteins from the RNA of these viruses must be by a process similar to that established for picorna-viruses - ie production of a giant polyprotein which is co- and post-translationally cleaved to produce the mature viral proteins.

7.2.2 Translating open reading frames and alignment of the two sequences

The program "TRANSLATE" was used. This program translates any nucleotide sequence into its corresponding amino acid sequence. For the Osloss sequence, the two ORFs were translated into separate files then joined head to tail to form a contiguous protein sequence. This protein sequence was aligned with the translated NADL ORF using the comparison program "GAP".

The amino acid sequences of Osloss and NADL are 3974 and 3988 residues long respectively. These were aligned using the comparison program GAP, which aligns two sequences to find the best match, inserting gaps where necessary in order to achieve this. GAP can only allow a comparison where the product of the characters is less than 1×10^6, eg 1000 x 1000 or 10 x 1000,000. The comparison of the two sequences was therefore split into sections of 1000 residues long.

The overall homology between NADL and Osloss at the protein level is about 90%. However, this homology was not evenly distributed: individual proteins and parts of proteins had different homologies, which are discussed below.

p116 is the precursor protein of the two putative structural glycoproteins p62 and p53. This region is one of the most variable, although the variation is localised mainly in p53, while p62 is 95% conserved. p53 is only 82% conserved. The number and position of the N-linked glycosylation sites (motif = Asn-X-Thr/Ser) are conserved. p62 possesses ten such sites and p53 possesses four. That p53 is the most variable of the pestivirus-coded proteins is not surprising since this protein has been identified as being involved in virus neutralisation. The C-terminus of p53 marks the end of the proposed structural proteins and also the end of the first ORF in Osloss. (Following p53 and before the first non-structural protein, there is an extremely hydrophobic stretch of 150 amino acids to which no protein has been assigned.

The proteins encoded C-terminally of p53 are considered non-structural. This region begins with the p125 precursor which, in NADL, and presumably Osloss, is processed to yield p54 and p80. The sequence of p54 is well conserved overall but both NADL and Osloss contain unique inserts at slightly different sites in this region. NADL contains a 90 residue insertion between amino acids 199 and 200 of the second Osloss ORF, while Osloss contains a unique insertion between residues 1679 and 1680 of the NADL genome. This insertion in the Osloss strain is identical in protein sequence to human ubiquitin, with one mismatch. Insertions in this region have now been demonstrated in another BVDV strain, CP1, and are correlated with cytopathogenicity (see discussion).

In p80, there are blocks of sequences which are conserved between flaviviruses, pestiviruses and eukaryotic helicase proteins. There are five such regions in p80. Bazan *et al* (1989) have in addition detected a trypsin-like serine protease domain in p80, the sequences making up this domain are highly conserved.

The final coding domain of NADL is represented by p133, a precursor processed to yield p58 and p75. p58 begins with good homology which becomes less evident as we approach the C-terminus. Within this region, however, there is a short stretch of amino acids which are also conserved in flaviviruses.

At the start of p75, there is a region of 40 amino acids which are variable, followed by a conserved region of 300 residues, another 60 residue variable region and a reasonably well conserved C-terminal sequence. There are two short stretches of homology with flaviviruses in p75.

7.2.3 Comparison of BVDV NADL and Osloss at the nucleic acid level

The entire 12kb of nucleotide sequences of the two strains were compared (in 12 x 1000 segments) using the program GAP. The overall homology was 75% and the distribution of conserved

areas followed the pattern of the amino acid sequence. The longest stretch of nucleotide homology was a 125bp segment spanning the 5′ untranslated region and the N-terminal of p20. Other particuarly conserved stretches were seen in regions corresponding to p62 (35bp long) and p80 (42bp long).

7.2.4 Comparison of the untranslated regions (UTRs)

The 5′ UTRs of NADL and Osloss show a homology of 90%, as opposed to the overall homology of 75%. This probably reflects the fact that these sequences have a functional role in recognition and control, for example, in replication and translation of the +-stranded genomic RNA. In both strains there appears to be a pseudo-start codon followed 4bp later by the authentic ATG. Interestingly, a similar pattern is also found in picornaviruses, though not in flaviviruses.

The 3′ UTRs are also 90% conserved for the last half of their length, but the first section (up to 12390) is only 70% conserved. This section includes an insertion in NADL of 38bp relative to Osloss. The largest totally conserved sequence in the 3′ region is a 35bp stretch.

7.2.5 Repeat sequences

The programme "REPEAT" was used to define direct and inverted repeat sequences in the UTRs of the two strains. There is a conserved direct repeat motif GGGTAG-4bp-CAGTGGT 42bp apart in the 5′ UTR of both strains. There are other, shorter, direct repeats conserved whose spacing varies between the two strains. There was also an inverted repeat sequence in the 5′ and 3′ UTRs as follows: ATA GGG TGC TGC AGA GC, which was fully conserved in both strains. This sequence is likely to be involved in the replication of the RNA.

The sequence TGTATATA is repeated 28bp apart in the 3′ UTR of NADL and 19bp apart in the 3′ UTR of Osloss.

7.3 COMPARISON OF BVDV SEQUENCES WITH HCV AND FLAVIVIRUSES

With the availability of the HCV Brescia sequence further comparisons with the BVDV sequences became possible. Reports have since been published (Moorman *et al*, 1990) of such a comparison. The sequence of yellow fever virus (YFV) was used as a flavivirus sequence (Rice *et al*, 1985).

7.3.1 Amino acid comparison

NADL, Osloss and HCV Brescia all share a high degree of homology and a very similar arrangement of the coding regions. The sequence of proteins is the same for HCV as for BVDV. There is about 70% homology between NADL or Osloss and HCV Brescia. The most notable differences between the HCV protein sequence and the two BVDV sequences was that HCV lacked any insertions in the p54/p80 region. The regions in p80 which were identified as motifs found in helicases and those forming the serine protease domain were also conserved between HCV and BVDV.

In the non-coding regions of HCV, the inverted repeat described for Osloss and NADL is also fully conserved lending further credence to the possibility that it is involved in RNA replication.

The protein sequences of pestiviruses have been compared to those of flaviviruses (Collett *et al*, 1989) and therefore this thesis did not include such comparisons. However, the small areas of homology which were described in the above report, were checked for homology with BVDV Osloss. These were completely conserved, and in fact the longest stretch of flavivirus homology in the BVDV strains forms part of the most conserved region in the p80 protein (corresponding to flavivirus protein NS3).

Comparison of the UTR regions revealed no similarities between pestiviruses and flaviviruses.

Flaviviruses have a similar arrangement of proteins to pestiviruses but when hydrophobicity plots were performed there was virtually no similarity.

7.4 CONCLUSIONS

The molecular features of BVDV were examined at the protein and nucleic acid level by using computer-assisted analysis. Two strains of BVDV, NADL and Osloss, were compared with each other, with HCV and with flaviviruses.

Homology at the protein level was very good between BVDV and HCV, about 70%, and of course, higher (87%) between the two BVDV strains. Sequence homology was virtually non-existent between pestiviruses and flaviviruses although a common protein arrangement seemed to occur. Both flaviviruses and pestiviruses would seem to share the same mechanism of gene expression, proteins being co- and post-translationally processed from a giant polyprotein transcribed from the single polycistronic mRNA. This processing may be done in part by cellular protease enzymes and in part by virus-encoded proteases. There have been putative protease cleavage sites identified (Moorman *et al*, 1990). These occur between amino acids 267/268, 560/561 and 689/690. These sites are conserved between the four pestiviruses (two BVDV, two HCV) so far examined. They correspond to the C termini of p20, p48 and p25 respectively.

The cleavage point in the non-structural precursor p125 occurs around position 5630 in BVDV (NADL). This precursor is only cleaved in cytopathic pestivirus strains and remains intact in non-cytopathic strains (Donis and Dubovi, 1987). The fact that p80 itself contains likely sequences for serine protease activity raises the possibility that it is a self-cleaving protein, which also contains a helicase activity. There is speculation as to whether the inserts observed in cytopathic virus strains around the cleavage point of p54/p80 are relevant to the way in which the p125 protein is processed, although there is no sound evidence as yet on which to base this.

Collett *et al* (1988a) have proposed, on the basis of sequence organisation and protein arrangement of the pestiviruses, to incorporate these viruses into the Flaviviridae family as a

separate genus. They argue that the alignment of proteins is sufficiently distinct to distinguish pestiviruses from the Togaviridae where they are currently classified. Both groups which have reported HCV sequences argue against the inclusion of pestiviruses in the Flaviviridae, due to the almost complete lack of sequence homology between the two groups, although they would accept such a reclassification were functional similarities found between pestivirus and flavivirus proteins.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

This thesis has presented work done at the molecular level on BD and BVD viruses.

At the beginning of this project, there was only one report in the literature describing the molecular cloning of a pestivirus sequence (Renard *et al*, 1985). This published sequence was for the Osloss strain of BVDV. During the course of this work, the interest and expertise in pestivirus molecular biology have gathered momentum, and there now exists sequence data for two BVDV strains, Osloss and NADL, and two HCV strains, Brescia and Alfort (Renard *et al*, 1985; Collett *et al*, 1988c; Meyers *et al*, 1989; Moorman *et al*, 1990). The organisation of the coding regions of the NADL strain (Collett *et al*, 1988b) was deduced, and proteins mapped to regions of the genome. This was a major step forward in terms of molecular knowledge of these viruses. Sequences of the four pestiviruses have been compared with each other and with flaviviruses (Moorman, 1990). A portion of the BDV-CP genome has been sequenced (Moerlooze *et al*, 1990). These workers used the PCR technique to sequence the carboxy-terminal portion of the 54K protein of eight pestivirus strains, including the BD-CP strain. The region sequenced was a conserved cysteine-rich area very similar to sequences found in proteins which have the zinc-finger configuration of nucleic acid binding proteins.

In this area at least, Border disease virus (CP strain) does not differ significantly from the NADL strain of BVDV (13 changes in 225) and was in fact more similar to BVDV-NADL than was BVDV Osloss (28/225) or HCV Alfort (61/225).

In addition, no significant differences were found between CP and NCP pestivirus strains, thus indicating that other areas of the genome must contribute to the cytopathic phenotype of these viruses.

The initial aim of this work was to produce a cDNA library from purified BDV RNA. This was attempted, as will be discussed shortly, but, in the course of this work other approaches were exploited, which gave results in areas not envisaged in the initial project plan. Such areas included studies on BDV replication, PCR work and computer-assisted analysis of existing pestivirus sequences.

The main aim initially, though, was to generate cDNA clones from the genome of BDV. This required growth and purification of large amounts of virus, isolation and purification of the viral RNA for use as a template, a method of priming cDNA synthesis on the template, a cloning system for cDNA thus generated, and a suitable method for screening clones for BDV sequences. In addition to the usual criteria to be satisfied, there were also special considerations and problems associated with pestiviruses in general, one probable reason for the dearth of reports on pestivirus molecular biology.

Pestiviruses are notoriously difficult to obtain in large purified amounts. They grow poorly and unpredictably in tissue culture, a fact borne out by results reported in Chapter 3, where titres of cultures of BDV could vary by 3 logs for no apparent reason. This meant that it was impossible to achieve a consistently efficient virus growth programme. In retrospect, it was realised that the scaling up of virus growth was not necessarily the best approach to large amounts of virus; BDV seemed to grow far better, and more consistently, in sealed plastic tissue culture flasks than in the large glass roller bottle cultures used in this work. Using glass, titres ranged between 10^3-10^6 $TCID_{50}$/ml, but in plastic flasks they averaged between 10^6 and 10^7/ml. Experiments described in Chapter 3 covered attempts to increase viral growth by using the drug Actinomycin D, which had been proposed to enhance the growth of BVDV NCP strains (Nuttall, 1980). The results reported in this chapter show an effect interesting in its own right, that AcD enhanced the growth of NCP and CP strains of BDV during the early stages of infection, but the effect was transient and

could not be utilized to increase yields of virus on a bulk scale. This difficulty in producing a high quality stock of virus for cloning purposes was undoubtedly the key factor in the failure to produce cDNA clones of BDV as described in Chapter 4.

The purification and handling of BDV genomic RNA was also an area of potential difficulty, firstly because it was available in such tiny amounts, and secondly because its length makes it extremely liable to attack by RNAases.

Various methods have been tried to minimise or circumvent these problems by the groups who have cloned pestivirus sequences. In the earliest report (Renard *et al*, 1985), BVD virus was purified from tissue culture supernatant. This group reported that the virus was present in the tissue culture fluid at a titre of 10^8pfu/ml and they used 3 litres of such fluid. Since this was the only published method for cDNA synthesis on pestiviruses available at the beginning of this work, we committed ourselves to a virus growth programme based on this report. This meant our target volume was at least 30L, since BD virus was known to grow to 1 log less than BVD virus.

A different approach entirely was taken by the group who sequenced BVDV-NADL (Collett *et al*, 1989). They found it impossible to purify enough high quality BVDV RNA from supernatant virus, so they isolated total RNA from virus-infected cells and purified the virus RNA fraction by precipitation in 2M $LiCl_2$, exploiting the fact that, because of areas of secondary structure, the viral RNA remained in the soluble fraction. Using this approach, enough pure viral genomic RNA was obtained to be visible on an ethidium bromide stained gel. In retrospect, this seems a better approach to obtain BDV RNA, assuming it has the same properties as the BVDV RNA.

The HCV cDNA cloning was also performed using intracellular viral RNA as a template. This group reported that HCV grew to a titre of 3×10^7 $TCID_{50}$/ml, and the cellular RNA fraction was

enriched for HCV sequences by centrifugation in a denaturing sucrose gradient.

Thus, if further attempts were to be made to construct a cDNA library from BDV, the method of choice would seem to be growth of virus in large plastic tissue culture flasks and isolation of total RNA from the infected cell monolayers followed by enrichment of the viral RNA fraction either by gradient centrifugation or $LiCl_2$ precipitation.

It would be extremely interesting to obtain sequence data from BD virus, since this is the only remaining pestivirus for which there is not complete molecular information. We can speculate that the overall organisation of the genome will be similar to BVDV and HCV, but the degree of sequence homology would be interesting to know. Antigenically all three pestiviruses are quite closely related, sharing a common soluble antigen. Neutralisation tests have shown BDV to be more related to BVDV- NADL than to HCV (Laude and Gelfi, 1979). Epitope mapping of the 53K glycoprotein allowed separation of BVDV strains into groups, NADL being the sole member of its group.

When BVDV and HCV protein sequences were compared, the two HCV sequences were 93% similar, the two BVDV sequences were 87% similar. HCV Alfort is also 85% similar to BVDV-NADL, not much more divergence than between BVDV strains. However, the Brescia strain of HCV is only 70% similar to either NADL or Osloss. It would be intriguing to know how similar BDV is to the BVDV and HCV strains. Areas of homology could be used to predict many features of the proteins from their sequences, for example, structural and functional domains, tertiary structures and active sites. Preliminary studies of this type have already been performed on BVDV sequences. Linear sequence motifs have been identified which strongly suggest the function of particular proteins, or domains within proteins. The amino acid sequence GDD is conserved amongst all RNA-dependent RNA polymerases so far sequenced, and this sequence is present uniquely within the

BVDV genome in the p75 protein (Collett *et al*, 1988b). This sequence also occurs in the HCV p75 protein (although it is not unique in the HCV genome), therefore there is a strong possibility that p75 is the viral RNA polymerase, or a part of it. Other predictions have been made on the basis of BVDV protein sequence data; the motif G X S G X P has been found in the N-terminal portion of p125, this sequence is similar to the motif conserved around catalytic serine residues of chymoptrypsin-like proteases. This has been taken one step further, comparison of the linear sequence of the p125 protein and the corresponding flavivirus NS3 protein has allowed a tentative prediction that the substrate-binding pocket involves conserved sequences downstream from the catalytic site. In the C-terminal section of p125, there are sequences similar to helicase (triphosphate-binding protein) sequences from a number of prokayotic and eukaryotic proteins. Thus, p125 appears to consist of two functional domains which can be cleaved to produce two separate proteins in cytopathic virus strains (Gorbalenya *et al*, 1989). The p54 protein of pestiviruses (the N-terminal section of p125) has been shown to possess a conserved cysteine-rich stretch of amino acids very similar to the "zinc-finger" sequences of nucleic acid binding proteins. These sequences are characteristic of proteins which interact directly with nucleic acid, and have been described for non-structural proteins of other positive-stranded RNA viruses. Helical prediction showed a conserved helix following the zinc- finger sequence. This conformation is found only in the cleaved p54 protein, and not in the intact p125, ie only in cytopathic virus strains (Moerlooze *et al*, 1990).

The experiments reported in Chapter 4 show preliminary results that the BDV genomic RNA is, like the other pestivirus RNAs, around 12kb long. It was also seen that the proportion of viral RNA in infected cells is very small. This is in accordance with observations on the other pestiviruses.

The replication mechanism of pestivirus RNAs in the infected cell is an area which remains unelucidated. No subgenomic RNAs have ever been detected in Northern blots either in this work or with BVDV (Renard *et al*, 1985). The degree of secondary structure present in the large genomic RNA would make it difficult to distinguish replicative forms or replicative intermediates. Since pestivirus replication is uninhibited by AcD, they do not rely on host cell enzymes for their RNA synthesis. They must, therefore, encode an RNA-dependent RNA polymerase which is translated directly from the +-stranded genomic/messenger RNA. The replication strategy for the +-stranded pestivirus RNA is unknown, but they would appear more similar in this respect to both flaviviruses and picorna viruses, neither of which synthesise sub-genomic RNA during replication. Alphaviruses on the other hand synthesis two sub-genomic RNAs, and three replicative intermediate (RI) molecules are clearly identifiable. Picornavirus replication has been well defined by both <u>in vivo</u> and <u>in vitro</u> studies (for review see Wimmer and Kuhn (1987)). The positive template strand after a few rounds of translation switches to being used as a template for minus strand RNA synthesis. Plus and minus strands are synthesised throughout the replication cycle, the former at about 10-fold excess. There have been RI molecules identified which are involved in plus strand synthesis. These molecules consist of template RNA with several nascent RNA chains attached. The mechanism for plus and minus strand synthesis may be different, as they appear to be under separate control. An important aspect of picornavirus repliction is that host cell proteins unrelated to nucleic acid metabolism may be recruited by the virus during RNA replication (see above review).

Flavivirus replication has also been well studied although not as thoroughly. For review see Brinton (1987). Like picornavirus there are no subgenomic RNAs detectable, and the polymerase gene maps to the 3′ end. The ratio of plus to minus strand RNA is again about 10:1. Both RI and replicative form (RF) structures have been detected. RNA synthesis is biphasic,

with synthesis peaking 6hrs after infection then a steady increase starting 13hrs after infection. Again it was suggested that minus strand synthesis may be under different control from plus strand synthesis.

Pestiviruses probably have a replication strategy very similar to picorna- and flaviviruses, ie a few rounds of translation, then synthesis of minus strand RNA followed by plus strand synthesis to produce mRNA and genomic RNA. The involvement of host cell factors is not unlikely, as has been observed for picornaviruses.

The results reported in Chapter 3 demonstrate that BD viral RNA synthesis has commenced by 4.5hrs post infection. Preliminary results showed a dramatic drop in RNA synthesis after this time, a phenomenon also reported by Nuttall (1980) for BVDV. This result would need to be repeated to assess its significance. If it is a true effect, it could be due to either release of labelled genomic RNA as free virus, or to incorporation of the label into a negative template RNA fraction on which the genomic RNA would be transcribed, and which would later be degraded. Further experiments designed to study the timing and partitioning of radiolabelled precursors could help shed light on the replication mechanisms of this virus.

The polymerase chain reaction (PCR) is a new technology which became available during this project. The primary requirement for a successful PCR is sequence information for making oligonucleotide primers. Several such primers were obtained and tested on BD virus, the sequences had been deduced by finding conserved areas on the BVDV NADL and Osloss sequences. The results of PCR are reported in Chapter 6. Consistent results were not obtained on BD virus RNA but some amplification was achieved. Amplification was excellent using BVDV RNA. This seems the most promising way to obtain sequence information on BD virus. Once a segment has been amplified and sequenced it can then be used as a probe to screen a cDNA library for adjacent sequences, and in this way, various amplified fragments could be

used to obtain sequence information either directly or by their subsequent use as probes.

This is one area where PCR could possibly be used to investigate replication kinetics. Primers designed to amplify either the plus strand sequence or the corresponding minus strand sequence could be used to quantify the amounts of each strand at any given time during virus infection, hence strand-specific primers could therefore be used to obtain ratios of plus strand to minus strand during the course of virus infection, which would help elucidate the mechanism of virus replication.

One intriguing question which could be tackled with the availability of sequence data for BD viruses is that of cytopathogenicity and the pathogenesis of mucosal disease in sheep. In cattle, a model for the onset of mucosal disease has been established, ie that the trigger for MD seems to be superinfection of a persistently infected animal with a CP virus which is antigenically similar to the persistently infecting strain (Howard *et al*, 1987). If pairs of viruses (CP and NCP) could be isolated from experimentally infected cases of mucosal disease and regions involved in antigenic variation (for instance the p53 region) were amplified and sequenced by PCR then sequences of different strains of CP and NCP viruses could be compared very quickly and the combinations giving rise to mucosal disease identified.

Another area where PCR sequencing would be useful is to compare sequences of the p125 protein in CP and NCP strains of BDV. Like BVDV, BDV possesses a protein which is cleaved to 80K in CP strains but is unprocessed in NCP strains (Dutia *et al*, 1990). It has been noted that the three CP BVDV strains sequenced so far have an insert in the p80 region, although the inserts differ in sequence and position. It would be interesting to compare CP and NCP BDV strains to determine if an insert in p80 was always associated with cytopathogenicity. This approach has already been taken by Meyers *et al* (1991) who found that

in addition to the insertions already discussed, a BVDV strain CP1 had an insertion of a complete p80 gene plus ubiquitin sequences, while its NCP "partner" had no insertions. Furthermore, they showed that p80 was expressed from this duplicated gene and not from p125, which was not processed in this strain.

In summary, this thesis describes work carried out at the molecular level on BDV and BD viruses. BD virus was grown in bulk and RNA produced from it. Attempts to synthesis cDNA from this template were unsuccessful and in that respect the original objective of the work has not been achieved. However, the work allowed a full insight into the problems associated with pestivirology on the practical level. We now have a much clearer idea of the limitations of the approaches tried, and where problems may be avoided in the future. Other work carried out during the project was more fruitful. The growth and replication of BDV was studied and a time course produced. Radiolabelled BDV genomic RNA was isolated from infected cells and shown to be 12kb long, in agreement with other data presented from ethidium bromide stained gels. Hybridisation studies were carried out using BVDV clones, one of which seemed promising in dot blot hybridisations. Finally PCR was used, a technique which holds much promise for elucidation of sequences, the ultimate aim of this work on BD virus.

With the current acceleration of pestiviral molecular biology many of the previously unanswered questions may now become accessible to being tackled at the molecular level. The more strains for which sequence data becomes available, the easier it will be to assign functions to proteins and to deduce replication mechanisms. The pivotal question of the mechanisms operating in mucosal disease may become clearer as more pestiviruses are defined at the molecular level.

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